Functional Characterization of Chromatin-Associated Protein ADP-Ribosylation

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Summary

Protein ADP-ribosylation is a post-translational modification (PTM) that consists of the addition of ADP-ribose moieties to target proteins. In the nucleus, the modification is catalyzed by members of the diphtheria toxin-like ADP-ribosyl-transferases (ARTDs), of which ARTD1 is the nuclear most abundant and best studied. ADP-ribosylation has been implicated in a variety of cellular processes, ranging from maintenance of genome integrity and DNA repair and gene transcription. However, very little is known about the localization and molecular function of chromatin-associated ADP-ribosylation.

In this thesis, we have investigated the mechanistic role of ARTD1-mediated chromatin poly-ADP-ribosylation (PARylation) for different cellular conditions. In the first paradigm, we investigated the role of ADP-ribosylation for the transcription of SOX2 target genes during the early phase of fibroblasts reprogramming to induced pluripotent stem cells (iPSC). We could show that ARTD1 and PARylation are necessary for iPS colony formation and that PAR formation keeps SRY (sex determining region Y)-box 2 (SOX2) associated to the DNA to efficiently transcribe SOX2 target genes, including *fibroblast growth factor 4* (*Fgf4)*. We found that exogenous FGF4 administration functionally rescues ARTD1 ablation or inhibition of PARylation. In addition, we have developed a novel chromatin affinity precipitation (ChAP) protocol to enrich ADP-ribosylated chromatin for elucidating where ADP-ribosylation is localized. We have applied this protocol to an oxidative stress paradigm and found that ADP-ribosylation induced by hydrogen peroxide (H_2O_2) preferentially localizes to ARTD1 and nucleosome-dense heterochromatic regions, as well as to repetitive elements within the genome. Chromatin ADP-ribosylation induced at these sites was correlated with a higher accessibility. Moreover during *in vitro* induced adipogenesis, ADP-ribosylation was tightly associated with peroxisome proliferator-activated receptor (PPARγ) at PPARγ target genes.

Together, the thesis reveals that chromatin-associated ADP-ribosylation is a PTM whose induction and genomic distribution varies with the stimulus and the cell type. Thus, for all tested conditions targeted ADP-ribosylation to defined chromatin loci either functionally regulated chromatin structure (as for H_2O_2) or gene transcription (SOX2 and PPARγ). How the target specificity of ADP-ribosylation in the chromatin context is achieved needs further investigation.

Zusammenfassung

Protein ADP-Ribosylierung ist eine post-translatare Modifikation bei der ADP-ribose an Zielproteine angehängt wird. Die verantwortlichen nuklearen Enzyme werden ADP-ribosyl-transferases diphteria toxin like (ARTDs) genannt, wobei ARTD1 am besten beschrieben ist. Die zellulären Prozesse bei denen ADP-Ribosylierung involviert ist, reichen von der Erhaltung der Genomintegrität und der DNS Reparatur bis hin zur Gentranskription. Dennoch ist sehr wenig über die Lokalisation und die molekulare Funktion von Chromatin-assoziierter ADP-Ribosylierung bekannt.

In dieser Arbeit haben wir die mechanistische Rolle von ARTD1 erzeugter poly-ADP-Ribosylierung (PARylierung) unter verschiedenen zellulären Konditionen untersucht. Im ersten Paradigma untersuchten wir die Rolle der ADP-Ribosylierung von SOX2 Zielgenen während der frühen Phase der Fibroblasten Reprogrammierung in induzierte pluripotente Stammzellen (iPSZ). Wir konnten zeigen, dass ARTD1 für die Formierung von iPS Zellkolonien nötig ist und dass PARylierung von SOX2 wichtig ist, damit *Fgf4* effizient transkribiert wird. Die exogenen Zugabe von FGF4 kompensierte die Inhibierung von PARylierung oder die Ablation von ARTD1. Des weiteren haben wir ein neues Chromatin Affinität Präzipitations Protokoll (ChAP) entwickelt, womit ADP-Ribosyliertes Chromatin angereichert werden kann und die Lokalisation der ADP-Ribosylierung geklärt werden kann. Wir haben dieses Protokoll auf ein oxidatives Stress Paradigma angewandt und gefunden, dass Wasserstoffperoxid (H_2O_2) induzierte ADP-Ribosylierung vorzugsweise zu Nukleosom-dichten heterochromatischen Regionen sowie zu repetitiven Elementen im Genom lokalisiert. Die an diesen Loci induzierte Chromatin ADP-Ribosylierung korrelierte mit einer höheren Zugänglichkeit des Chromatins. Zusätzlich war während der *in vitro* induzierten Adipogenese, die ADP-Ribosylierung eng mit PPARγ bei dessen Zielgenen assoziiert.

Zusammengenommen zeigt diese Arbeit, dass die Induktion und genomische Distribution von Chromatin-assoziierter ADP-Ribosylierung vom Stimulus und Zelltyp abhängt. Unter allen getesteten Konditionen hat die zielgerichtete ADP-Ribosylierung zu bestimmten Chromatin Loci entweder die funktionelle Chromatin Struktur (bei H_2O_2), oder die Gentranskription (bei SOX2 und PPARγ) reguliert. Wie die zielgerichtete Spezifität der ADP-Ribosylierung im Kontext von Chromatin erreicht wird bedarf weiterer Untersuchungen.

Table of Contents

Abbreviations

aa	Amino acid
ADPr	Adenosine diphosphate ribose
ARH	ADP-ribosyl hydrolase
ART	ADP-ribosyltransferase
ARTD	ADP-ribosyltransferase diphtheria toxin-like
ARTC	ADP-ribosyltransferase cholera toxin-like
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BER	Base excision repair
bp	Base pair
BRCT	BRCA1 carboxy-terminal domain
C/EBP	CCAAT/enhancer binding protein
ChIP	Chromatin immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DSB	Double-strand break
dsDNA	Double-stranded DNA
ES cells	Embryonic Stem cells
FCS	Fetal calf serum
FGF4	Fibroblast growth factor 4
HDAC	Histone deacteylase
H_2O_2	Hydrogen peroxide
H3K4me1	Histone H3 lysine 4 mono-methylation
H3K4me2	Histone H3 lysine 4 di-methylation
H3K4me3	Histone H3 lysine 4 tri-methylation
H3K9me3	Histone H3 lysine 9 tri-methylation
H3K27Ac	Histone H3 lysine 27 acetylation
H3K27me3	Histone H3 lysine 27 tri-methylation
H3K36me3	Histone H3 lysine 36 tri-methylation

IBMX	3-Isobutyl-1-methylxanthin
IL6	Interleukin 6
iPSC	Induced pluripotent stem cells
LPS	Lipopolysaccharide
MARylation	Mono-ADP-ribosylation
MDO	Macrodomain containing protein
MEF	Mouse embryonic fibroblasts
NAD	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
nt	deoxyribonucleotides
PAR	Poly-ADP-ribose
PARG	Poly-(ADP-ribose)-glycohydrolase
PARP	Poly-(ADP-ribose)-polymerase
PARylation	Poly-ADP-ribosylation
PBS	Phosphate buffered saline
PNKP	Polynucleotide kinase-phosphatase
Pol II	Polymerase II
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPARγ response elements
PTM	Post-translational modification
RNA	Ribonucleic acid
RXR	Retinoid-X receptor
SOX2	SRY (sex determining region Y)-box 2
SSB	Single-strand break
TNFα	Tumor necrosis factor α
WAT	White adipose tissue
XRCC1/4	X-ray repair cross-complementing protein 1/4

1 Introduction

1.1 Chromatin

The genome is the entire set of genetic information of a certain organism. The human haploid genome is divided in 22 autosomal and one sexual chromosome and composed of 3.3 billion deoxyribonucleotides (nt) forming the DNA[1,2]. The genomic DNA is enclosed in the nucleus with the exception of a small molecule of mitochondrion DNA. The nuclear DNA, proteins and RNAs form a highly dynamic macromolecular complex named chromatin. The chromatin main functions are to fit the DNA in the nucleus, preserve the DNA integrity from harmful agents and to regulate the accessibility to the genomic information (e.g. during transcription)[1,2]. Chromatin is hierarchically organized. On the first level the naked DNA is wrapped around the first basic unit, the nucleosomes. Nucleosomes are composed of 8 histone proteins, the octameric nucleosome core, and a 147 bp long stretch of DNA wrapped 1.7 times around the nucleosome core. The nucleosome position is determined by several factors, including the DNA sequence, the presence of DNA-binding proteins, the action of ATP-dependent nucleosome remodelers, the transcription machinery, DNA-replication complex and repair factors[3-5]. The resulting structure is called the 10 nm fiber and corresponds to chromatin in open conformation (Fig.1)[6].

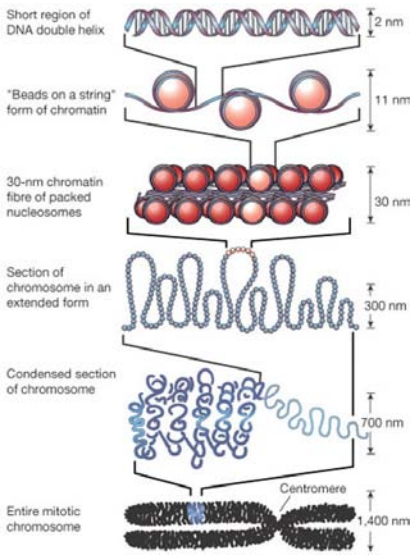

Figure 1: The chromatins hierarchy. Modified from[7] .

The 10 nm fiber is further folded into the 30 nm fiber by the addition of the linker histone H1. The 30 nm fiber is more compacted compare to the 10 nm fiber[8]. Ultimately, during the mitotic metaphase, when the duplicated genome has to be equally distributed between the 2 daughter cells, chromatin reaches its highest condensation in the chromosomes, structures that ensure the proper segregation of the genetic information[9]. The classical vision of the chromatin hierarchy as been recently challenged because, although some experimental evidences have proved the existence of the 30 nm fiber *in vitro*, other studies failed to detect such structure *in vivo* [10,11].

1.1.1 Histones

Histones are a family of basic proteins characterized by a common globular "helix turn helix turn helix" motif that facilitates their dimerization and two terminal tails, one at the N-terminus and the other at the C-terminus, that undergo extensive post-translational modification to regulate the nucleosome properties[6,12]. Histones are subdivided into five classes: histone H1, H2A, H2B, H3 and H4. Two H2A-H2B dimers and two H3-H4 dimers complexed together compose the octameric nucleosome core, whereas histone H1 binds the linker DNA within two adjacent nucleosomes and is responsible for nucleosome-nucleosome interaction and local chromatin compaction (see 1.1). In addition to the canonical histones, each histone class also encompasses a number of non-allelic variants commonly referred to as histone variants[13]. Histone variants diverge from the canonical histones due to mutations in their primary sequence that confer them different structural and functional properties. While canonical histones are primarily expressed in S-phase and deposited on the DNA in a replication-dependent manner, histone variants are expressed throughout the cell cycle and their distribution is restricted to defined genomic loci, tissues and development stages[13].

1.1.2 Histone post-translational modifications

Histones, like many other proteins, are post-translational modified when one or more amino acids are covalently linked to certain chemical groups. In the case of histones the two terminal tails are extensively modified, whereas the globular domain shows a lower extent of modification[14]. Among the many modifications, the most studied and relevant are acetylation, methylation, phosphorylation, sumoylation, ubiquitylation and ADP-ribosylation[15]. The enzymes responsible for the deposition of a certain modification are called "writers", the proteins that recognize and bind the

modification are called “readers”, whereas the enzymes that remove the modification are called “erasers”[16]. On one side, these modifications change the chemical and physical properties of the nucleosome and on the other side, they represent docking platforms for chromatin remodelers and transcription factors, important effector proteins that carry out specific biological processes. Since more than one histone post-translational modifications (PTM) can be deposited on the same nucleosome or on adjacent nucleosomes, the existence of a histone code has been postulated[17]. The histone code is a signature of PTMs that mechanistically define the function for a specific region of chromatin. For example, high levels of histone H3 lysine 4 mono-methylation (H3K4me1) and low amount of histone H3 lysine 4 di-methylation (H3K4me2) and histone H3 lysine 27 acetylation (H3K27Ac), in combination with the presence of lineage specific transcription factors, define active enhancer regions whereas, if histone H3 lysine 27 is tri-methylated (H3K27me3), it becomes a common signature of posed enhancers[18].

The euchromatin is characterized by the presence of transcription factors, low nucleosome density and enriched in histone marks such as histone H3 and H4 acetylation, histone H3 lysine 4 tri-methylation (H3K4me3) and histone H3 lysine 36 tri-methylation (H3K36me3)[19]. Instead, the heterochromatin is a nucleosome dense structure with histone marks such as histone H3 lysine 9 tri-methylation (H3K9me3), H3K27me3 and histone H4 lysine 20 methylation[20].

In this way PTM of histones and more generally chromatin-associated proteins define the chromatin landscape and fine-tune virtually all the processes in the nucleus[15].

1.2 Protein ADP-ribosylation

Protein ADP-ribosylation is a PTM generated by a class of enzymes named ADP-ribosyltransferases (ARTs) that uses the nicotinamide adenine dinucleotide (NAD^+) to covalently attach adenosine diphosphate ribose (ADPr) to a specific protein-residue and release a free molecule of nicotinamide (Fig.2)[21].

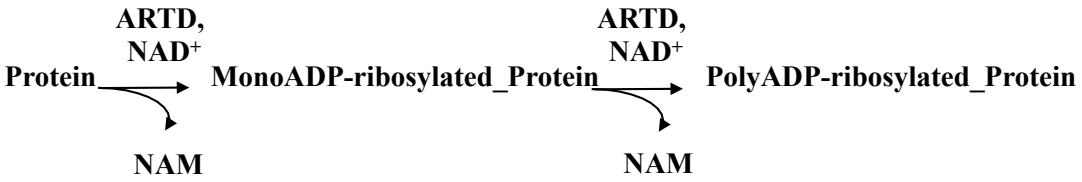

Figure 2: Schematic representation of ADP-ribose transfer reaction.

ADPr is a chemical structure that at physiological pH carries 2 negative charges and a high-energy pyrophosphate bond[22]. ARTs specifically modify extracellular, cytoplasmic as well as nuclear and chromatin-associated proteins including histones[23]. Several amino acids have been shown to be acceptors of ADPr: arginine, asparagine, glutamic acids, aspartic acid, cysteine and more recently lysine [23,24]. Since the first ADPr can be further extended by the subsequent addition of ADPr units, ADP-ribosylation can be either classified as mono-ADP-ribosylation (MARylation) or poly-ADP-ribosylation (PARylation)[25]. The half-life of protein ADP-ribosylation can be as short as few seconds or within the hour range, depending on the nature of the stimulus[26,27]. This is controlled by the activity of the ADP-ribose hydrolases that reverse the formation of ADP-ribosylation by degrading the polymers and removing the protein-bound ADPr moiety. Poly-ADP-ribose glycohydrolases (PARG) and ADP-ribosylhydrolase 3 (ARH3) were shown to hydrolase PAR whereas ARH1, the human macrodomain-containing protein D1 (MDO1), D2 (MDO2), C6orf130 and the archeabacteriae Af1521 are able to release the protein-bound ADPr from specific amino acids, rendering ADP-ribosylation a reversible modification[28-31].

1.2.1 Mono-ADP-ribosylation

MARylation was first discovered to be catalyzed by certain bacterial toxins. Cholera, diphtheria and pertussis toxins catalyze the MARlation of host proteins such as actin, elongation factor 2, Rho and Rac to inhibit their activity and contribute to the

pathological condition observed after bacterial infection[32]. More recently intracellular and extracellular MARlation has been described as regulator of gene transcription, inflammation and cell differentiation[31,33].

1.2.2 Poly-ADP-ribosylation

De novo protein PARylation is initiated with the transfer of an ADPr to a specific amino acid to generate a MARylated residue. This protein-bound moiety is then extended with a second ADPr through a glycosidic ribose-ribose bond. This elongation is repeated to give rise to an oligomer and eventually a polymer of ADPr. Polymers can be linear or branched and can reach a length of 400 moieties *in vitro*[34]. MARylation as well as PARylation change the behavior of the target protein in a manner that is dependent on the nature of the protein and on the context. PARylation has been described to modulate the activity of its targets in both directions: they can either been activated or inhibited[35-37]. In addition, PAR functions as a scaffold to recruit specific PAR-binding proteins, generating a complex protein network whose functions are still largely unknown[38-40]. PARylation has been reported to influence a growing number of cellular processes such as DNA repair, transcription, chromatin remodeling, RNA maturation and degradation, telomere maintenance, inflammation, stress response, cell differentiation and reprograming[25].

1.2.3 ADP-ribosyl transferases (ARTs)

The enzymes responsible for generating ADP-ribosylation are named ARTs and are subdivided into two classes according to the structural similarity of their catalytic domain to cholera (ARTCs) or diphtheria (ARTDs) toxin[41]. The members of the ARTC subfamily are mainly anchored to the cytoplasmic membrane acting in the extracellular space as mono-ADP-ribosyltransferases[41]. The ARTD family encompasses eighteen proteins (ARTD1-18) in human and seventeen in mouse that share a common catalytic domain[41]. A recent study, aiming at characterizing the activity of each member of the ARTD family, reported that ARTD1, 2, 5 and 6 are able to generate PAR, ARTD9 and 13 are inactive and all the other members are only capable of transferring a single ADPr to the target amino acid[42]. Nevertheless, contradictory reports have been published for some ARTDs. For example, it is not clear whether ARTD3 is a mono- or poly-ART[42-44]. The most abundant and most studied member of the ARTD family is ARTD1, formerly named poly-ADP-ribose polymerase 1 (PARP1). ARTD1 is an abundant nuclear and chromatin-associated

protein responsible for most of the nuclear poly-ADP-ribosylation[45,46]. Very long investigated for its role in DNA repair (see 1.5.3), ARTD1 has emerged more recently as a transcription regulator[25]. ARTD1 is recruited to both euchromatin as well as heterochromatin, modulating chromatin function in a context-dependent manner.

1.2.4 ADP-ribose binders and their application to enrich ADP-ribosylated proteins

ADPr binders are protein domains that bind to ADP-ribosylated proteins and act as "readers" of such modification. Four main classes of ADPr binders have been identified so far, including the macrodomain, the PAR-binding motif (PBM), the PAR-binding zinc-finger domain (PBZ) and the WWE domain (Fig.3)[25,47].

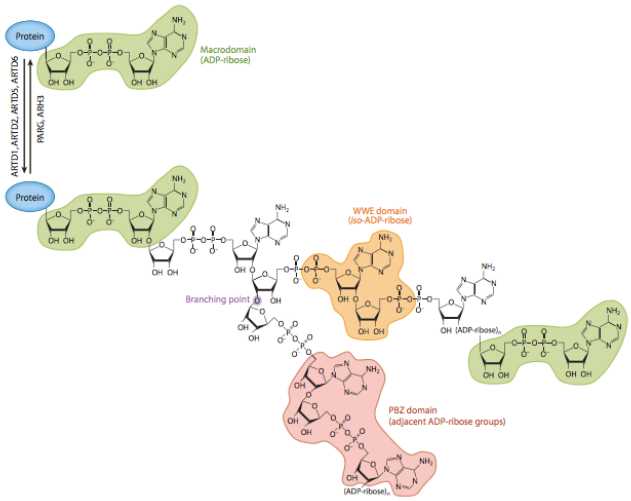

Figure 3: Representation of ADP-ribose binders specificity[25]

The madrodomains are evolutionary conserved protein modules sharing a high degree of structural homology[25]. Differences in the macrodomain structures account for their selective binding to MAR, PAR or both of them[48]. Some macrodomains are also able to hydrolase ADP-ribosylation and are therefore ADPr "erasers" (see above 1.1.2). The WWE domains are positive charged protein domains composed of a half β-barrel juxtaposed to a α-helix named after the 3 conserved amino acids in the primary sequence[49]. WWE domains recognize the smallest PAR structural unit, the iso-ADPr, and do not bind to MARylated proteins[25,49]. They are found in E3-ubiquitine ligases or ARTDs as a single domain or in tandem[41,50]. Both the PBM and PBZ recognize PAR but not MAR[25].

The high affinity of such domains for PAR or MAR makes them suitable tools for studying ADP-ribosylated proteins[51]. Indeed, in order to study protein ADP-ribosylation in the cellular context, it is necessary to isolate proteins that are modified from the ones that are not. Until recently, an anti-PAR antibody developed in the early 80's, named 10H, has been the most commonly used tool to investigate protein AD-ribosylation[64]. 10H is a mouse monoclonal antibody with high affinity for linear and long PAR (>20mer), moderate affinity for shorter polymers (10-30mer) and unable to recognize shorter oligomers of ADP-ribose or MARylation[52,53]. Although the 10H antibody is an important tool, the requirement of long polymers for its binding, limits its usage. Other anti-PAR antibodies, that recognize shorter polymers, have been recently developed and are now commercially available[54]. However, the exact epitope they recognize and whether extensively branched polymers are recognized as well is not yet clear[55,56]. To overcome these limitations recent studies have replaced the anti-PAR antibodies with ADP-ribose binders of different origins[57,58]. The macrodomains, for example, represent powerful baits that can be used to address whether a protein is PARylated or MARylated. Indeed, the Af1521 macrodomain from *Archeaoglobus fulgidus* has been recently used as bait to enrich for ADP-ribosylated proteins in large-scale mass spectrometry screenings aimed at defining the cellular ADP-ribosylome[57,58]. Other ADPr binders have been characterized for their capacity to bind ADP-ribosylated proteins. In particular the histone H2A1.1's macrodomain has high affinity for PAR whereas the ARTD9 macrodomains 1 and 2 recognize MARylated ARTD10[59,60]. The WWE domains bind to iso-ADP-ribose, the smallest PAR structure, and are therefore able to pull-down short oligomers as well as long PAR but do not recognize MARylated proteins[49]. Thus, the WWE domains can be exploited to discriminate PARylated proteins irrespectively of the length or structure of the modification. Another strategy to isolate ADP-ribosylated proteins takes advantage of resins coupled to boronic acid, which covalently binds to the cis-diol groups of the riboses in the ADPr moiety[56]. Although this technique has been used to identify ADP-ribosylated proteins in cell and tissue extracts, the boronate group does not only bind to ADPr, but also to other organic cis-diol groups, thus representing a potential source of bias[61,62]. An alternative approach relies on the permeabilization of cells and their incubation with NAD^+ derivatives such as biotinylated NAD^+ or $1,N^6$etheno NAD^+. Once in the cells, these compounds are used, although with lower efficiency, by ARTDs to modify target proteins[55,63,64].

The modified proteins can then be separated either by resin affinity purification or with specific antibodies. The drawback of these protocols is that the cell permeabilization step may induce biological artifacts and that the lower efficiency of incorporation may lead to an underestimation of the proteins that are ADP-ribosylated.

1.2.5 ADP-ribosylation in the chromatin context

Very little is known about chromatin-associated ADP-ribosylation *in vivo* and its interplay with other histone modifications or the chromatin context where it is formed. Recent studies on Drosophila have revealed that ARTD1 is recruited to the linker DNA between 2 nucleosomes of which one contains the canonical histone H2A, whereas the other incorporates the histone variants H2Av, the homologous of the human H2Ax and H2Az[65]. In this context, phosphorylation of H2Av serine 137 on one nucleosome and acetylation of H2A lysine 5 on the other promote PAR formation to induce nucleosome eviction and efficient gene transcription in response to heat shock[65]. Along the same line, ARTD1-mediated ADP-ribosylation has been reported to promote gene transcription in lipopolysaccharide (LPS)-stimulated murine macrophages by histone PARylation and subsequent nucleosome eviction from the promoters of NF-κB target gene[64,66]. However, other study have shown that, upon cell stimulation with LPS, ARTD1 recruits p65 and the histone acetyl-transferase p300 to reorganize the local chromatin and promote the transcription of NF-κB target genes in an ADP-ribosylation independent manner[67]. In addition, the human ARTD1 has been described to replace histone H1 at active promoters[68]. In line with these evidences, ARTD1 PARylates KDM5B to inhibit H3K4me3 demethylation therefore placing ADP-ribosylation in an open chromatin context and functionally linking it to gene transcription[36]. Conversely, after nuclear micro irradiation, ARTD1 activation influences the genomic localization of the human histone variant macroH2A1.1, an ADPr binder, to enhance local genomic compaction[59]. Furthermore, nuclear ADP-ribosylation has been implicated in the maintenance of rDNA repression after DNA replication, placing ADP-ribosylation in a completely different heterochromatic context[37,55]. In line with that, heterochromatin-associated ARTD1 and ADP-ribosylation has been reported to modulated UHRF1 and DMNT1 activity and to promote the re-establishment of rDNA silencing[37,69,70]. Collectively all these

evidences suggest that chromatin-associated ADP-ribosylation is restricted to defined genomic regions and that its functional outcomes are influenced by the chromatin context where it is formed.

1.3 Cell fate determination

Every cell in a complex organism originates from a single totipotent cell through a hierarchical process encompassing several steps of cell proliferation, fate commitment, cell specification and chromatin rearrangements that eventually lead to fully differentiated somatic cells[71]. These processes are subject to tight regulation and play a fundamental role during tissue development and regeneration and maintenance of body homeostasis. However, somatic cells can also be reprogrammed in order to re-acquire pluripotent or totipotent features. This is the case during gametogenesis, *in vitro* reprogramming or tumorigenesis and metastasis[72,73]. Interestingly, ADP-ribosylation has been reported to act in all the above-mentioned processes. For instance, depletion of the only Drosophila ARTD or mouse co-depletion of ARTD1 and ARTD2, the two prominent nuclear PAR-generating enzymes in mammals, have lethal effects at the larvae or embryonic stage respectively, suggesting that ADP-ribosylation is essential for proper development of multicellular organisms[74,75]. In addition, ARTD1 has been reported to PARylate SRY (sex determining region Y)-box 2 (SOX2), a major pluripotent transcription factor, in embryonic stem (ES) cells and promote cell differentiation[76]. Conversely, upon embryonic reprogramming of mouse germ line, ADP-ribosylation enhances the acquisition of genomic pluripotent features by erasing DNA methylation[77].

1.3.1 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSC) are pluripotent stem cells that originate from somatic cells through an engineering process[72]. In 2006, the Yamanaka laboratory discovered that retroviral transduction of fibroblasts with the transcription factors octamer-binding transcription factor 4 (OCT4), SOX2, Kruppel-like factor 4 (KLF4) and V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (c-MYC) (named the Yamanaka factors) was sufficient to reprogram somatic cells to pluripotent stem cells capable of differentiating to any of the three germ layers[72]. Despite the promising application in regenerative medicine, a better understanding of the molecular mechanisms that drive reprogramming is necessary, especially considering that iPSC are generated by stably overexpressing 4 oncogenes that may potentially give rise to

malignant cells. To overcome this risk, new protocols have been developed. Retroviral transduction of the Yamanaka factors has been replaced with transient transfection of these factors and their number was reduced to three or two, in combination with small molecule HDAC inhibitors[78]. The two factors that seem to be essential at the onset of reprogramming are OCT4 and SOX2. They act singularly or together to sustain self-renewal and pluripotency[78]. Interestingly, SOX2 not only regulates reprograming, but its fine-tuned expression is one of the determinants in keeping a stem cell pluripotent or pushing it into differentiatiation[79]. In particular, SOX2 has been implicated in neuronal differentiation and neuronal progenitor maintenance[80]. Mechanistically, SOX2 binds the promoter or regulatory regions of target genes and promotes their transcription as single transcription factor or in complex with OCT4 and NANOG[80]. This is also the case for the fibroblast growth factor 4 (FGF4), whose expression in the epiblast as well as *in vitro* cultured ES cells is driven by SOX2[81]. The expression of FGF4, in turn, induces the paracrine secretion of FGF4 sustaining trophoblast stem cell renewal and multipotency. SOX2 is thus a master regulator of reprogramming via fine-tuning complex pathways that are not yet completely understood. Full understanding of the reprograming process is needed to generate new powerful tools in regenerative medicine and to avoid the risk of cancer development in patients.

1.3.2 Adipogenesis

The adipose tissue is a complex network of cells, primarily adipocytes, with several functions, such as maintenance of body lipid homeostasis, body energy storage and regulation as well as adipokines and hormones production[109]. Its mis-regulation is linked to metabolic disorders such as diabetes, atherosclerosis, obesity and cancer[82,83]. Adipocytes are classified into white adipocytes and brown adipocytes[84]. Although these two classes of cells have different functions, they are tightly related. White adipocytes can trans-differentiate into brown ones and vice versa, rendering adipogenesis, the generation of adipocytes, a “two-way” process. White adipocytes, together with precursor cells, endothelial cells, immune cells and fibroblasts constitute the white adipose tissue (WAT) whose main functions are to insulate the body, generate a mechanical barrier to protect the body, store energy and to produce adipokines[84,85]. The brown adipose tissue (BAT) is responsible for lipid oxidation and heat generation, thus contributing to body temperature homeostasis[78]. Cell

differentiation into adipocytes is a complex process that can be recapitulated using a model cell line (3T3L1) *in vitro* [86]. During the early phase of differentiation, cells express the CCAAT/enhancer binding protein (C/EBP) -β and -δ, two key transcription factors that drive differentiation by promoting the transcription of C/EBPα and of the peroxisome proliferator-activated receptor γ (PPARγ) in the late phase[87]. The latter is a nuclear receptor that, in response to not yet fully characterized ligands, interacts with the retinoid X receptor (RXR) and is then recruited to PPARγ target genes promoting their transcription[87]. PPARγ target genes are characterized by the presence of *cis*-regulatory sequences named PPARγ response elements (PPRE), where PPARγ binds, bringing along the histone acetyltransferases p300-CBP[88,89]. Interestingly, ARTD1-mediated PARylation has been shown to increase during differentiation and ARTD1 ablation or inhibition of ADP-ribosylation both hampers adipogenesis *in vivo* and *in vitro*[90,91]. Mechanistically, ARTD1 is activated by topoisomerase-II (TOPOII) at PPARγ target genes where PAR formation enhances PPARγ ligand binding, which subsequently induces co-factor exchange (i.e. p300-CBP recruitment) and chromatin remodeling to stimulate gene transcription[92].

1.4 Oxidative stress and H_2O_2signaling

1.4.1 Oxidative stress

Oxidative stress is an imbalanced condition between the amount of free radicals in the cell and the cell antioxidant capacity[93]. Reactive oxygen species (ROS) are of environmental as well as of endogenous origin. For instance, during mitochondrial respiration, superoxide anions (O_2^-) are generated and need to be converted by the superoxide dismutase (SOD) to the less harmful hydrogen peroxide (H_2O_2) that is eventually transformed by catalases to H_2O[94]. H_2O_2 has been implicated in cell signaling and tissue regeneration[95-97]. For example, in response to tissue injury, epithelial cells produce H_2O_2 that acts as chemo-attractor for neutrophils and leukocytes to prevent possible infection[98]. In addition, H_2O_2 contributes to angiogenesis and epithelial cell migration by activating signal cascades such as ERK1/2 phosphorylation and NF-κB nuclear translocation to promote the transcription of pro-inflammatory genes and genes involved in wound healing, such as VEGF, IL8 and MUC1[99,100].

1.4.2 H_2O_2 signaling

The intracellular mechanisms that sense H_2O_2 and initiate the signaling cascades have not been fully identified, but it seems that metallo-enzymes as well as cysteine oxidation play a great role[101,102]. In particular, the cysteine thiolate ($-S^-$) is prone to be oxidized by H_2O_2 to sulfenic acid (-S-OH) and further to sulfinic ($S-O_2H$) and sulfonic ($S-O_3H$) acid and to form cysteine-cysteine bonds[101]. Each intermediate can be rapidly reversed when normo-conditions are re-established. H_2O_2 has not only been correlated with protein oxidation, but also with lipid oxidation as well as single strand (ssDNA) and double strand DNA (dsDNA) damage, leading to activation of DNA repair pathways, increased DNA mutations and cell death[103-105]. Although the exact mechanism by which H_2O_2 induces DNA damage is not completely understood, it seems that chromatin-associated iron mediates the formation of damages that can target either the nucleobases of the DNA, that may lead to DNA mutations if not properly repaired, or the sugar bonds, leading to the generation of ssDNA breaks that, if not efficiently repaired before DNA replication, may cause stalling of the DNA replication fork and eventually generate dsDNA breaks[106-108]

1.4.3 PAR in Base Excision Repair (BER) pathway

Oxidized DNA bases, such as 8-oxoguanines, are recognized and excised by the OGG1 glycosylase[109]. This reaction leaves the DNA with an abasic site, also known as AP site. The BER pathway is responsible for repairing DNA base lesions or ssDNA breaks[110]. The AP site is further processed by an AP endonuclease to generate a single strand nick. This intermediate is then bound by polynucleotide kinase-phosphatase (PNKP) to generate the proper 3' hydroxyl and 5' phosphate ends that are required for pol β and λ to fill the gap replacing the single nucleotide (short-patch BER) or for pol σ and pol ε to synthetize a larger stretch of DNA displacing the damaged strand (long patch BER). In this latter case, the displaced ssDNA must be removed by the FEN1 endonuclease. Eventually, both short and long patch BER pathways end with DNA ligase I or III that seals the nick[109]. Although it is well established that intracellular oxidative stress (e.g. treatment with H_2O_2) induces nuclear PAR formation, its role in BER is still under debate[111]. Some reports have shown that DNA lesions activate ARTD1 and that its activity is involved in recruiting XRCC1 to the damaged site[112,113]. In contrast, other reports excluded that ARTD1 and PAR have any implication in the first phases of BER, suggesting that ARTD1 may

only be activated to protect the damaged sites from nuclease attacks when the cellular repair capacity is exceeded[114,115]. Interestingly, in response to DNA damage or H_2O_2, ARTD1 is phosphorylated by ERK1/2 and JNK1, which leads to enhanced and sustained PAR formation, indicating that PAR formation is not a mere consequence of DNA damage detection by ARTD1 but rather the result of several regulated factors connecting PAR formation to cell signaling[116,117].

1.5 ARTD1 and ADP-ribosylation in inflammation

Due to the role of ARTD1 and ARTD2 in DNA repair (see 1.4.3 and [118]) inhibitors of ADP-ribosylation have been primarily used to enhance the anti-cancer effects of some DNA damage-inducing chemotherapies or radiation therapy in BRCA1/2 mutant breast tumor and in other malignant cancers[119,120]. However, inhibitors of ADP-ribosylation have been also considered for the treatment of other acute and chronic disorders such as cardiac, neuronal, metabolic and inflammatory diseases[120]. In line with that, inhibitors of ADP-ribosylation have shown protective effects in several inflammatory disorders, stressing the medical relevance of ADP-ribosylation in inflammation[121]. For example, murine macrophages treated with the inhibitor of ADP-ribosylation PJ34 upon exposure to LPS, showed reduced pro-inflammatory cytokines production compared to non-inhibited cells[64]. Furthermore, mice lacking ARTD1 are resistant to LPS induced endotoxic shock and release less TNF-α, IFN-γ and nitric oxide into the blood stream when compared to wild-type mice [122]. ARTD1 has also been implicated in T-lymphocyte differentiation and inhibitors of ADP-ribosylation have been reported to protect from excessive inflammatory activation, indicating that ADP-ribosylation may enhance inflammation and induce cell necrosis due to over-production of cytokines[121]. In addition, ARTD1-/- cells fail to activate a NF-κB dependent reporter gene and only the genetic complementation of cells with ARTD1 allowed successful gene expression[122,123]. These data suggest that ARTD1 functions in transcription as co-factor of NF-κB, even though it is still under debate whether ADP-ribosylation is directly involved in NF-κB target gene transcription[64,124,125]. Indeed, most of the inhibitors of ADP-ribosylation used to address the implication of ARTD1 enzymatic activity on NF-κB target gene transcription belong to the first or second generation of inhibitors, which may have off-target effects[25,126].

2 Aim of the thesis

ADP-ribosylation is a protein PTM linked to a broad variety of physiological and pathophysiological processes such as tissue development, inflammation and cancer onset or progression. Nuclear ADP-ribosylation regulates the intracellular energy metabolism, stress response and cell death by locally rearranging chromatin and influencing DNA repair and gene transcription. However, due to the lack of suitable methodologies, only little is known about the molecular mechanisms that induce chromatin-associated ADP-ribosylation and its functional implications.

Our hypothesis was thus ARTD1 is locally activated on the chromatin to mediate the ADP-ribosylation of chromatin-associated proteins and support gene transcription. Aim of this thesis was to investigate the functional contribution of ARTD1 and chromatin ADP-ribosylation on gene transcription. We approached the biological question by:

a) Addressing how ARTD1-mediated ADP-ribosylation sustains the early phase of fibroblasts reprogramming to iPSCs.
b) Using H_2O_2-induced PAR formation to develop a novel method that allows the detection of chromatin-associated ADP-ribosylated proteins and applying it to investigate PAR formation during adipogenesis.
c) Investigating how ARTD1-mediated ADP-ribosylation influences the transcription of NF-κB target genes.

3 Results

3.1 Published results

Artd1/Parp1 regulates reprogramming by transcriptional regulation of Fgf4 via Sox2 ADP-ribosylation.

Authors: Weber FA, **Bartolomei G** (first co-authorship), Hottiger MO,Cinelli P.
Journal: Stem Cells, 2013
Contribution: Planning, performing, evaluating experiments for: Fig.1C-F, Fig.2, Fig.3B and Supplementary Fig.3. writing and editing manuscript.

3.2 Submitted manuscript

A new highly specific ChAP method for the genome-wide localization of chromatin ADP-ribosylation.

Authors: **Bartolomei G**, Hottiger MO.
Journal: Submitted
Contribution: Planning, performing and evaluating all the experiments; figures preparation; writing and editing manuscript.

3.3 Unpublished results

3.3.1 H_2O_2-induced ADP-ribosylation does not change chromatin composition and is associated with constitutive heterochromatin

3.3.2 H_2O_2-induced ADP-ribosylation or inhibition of ADP-ribosylation do not influence NF-κB-dependent gene expression

3.1 Published results

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Artd1/Parp1 Regulates Reprogramming by Transcriptional Regulation of Fgf4 Via Sox2 ADP-Ribosylation

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Key Words. Cell biology • Induced pluripotent stem cells • Artd1/Parp1 • Pluripotent stem cells • Reprogramming • Fgf4

ABSTRACT

The recently established reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) by Takahashi and Yamanaka represents a valuable tool for future therapeutic applications. To date, the mechanisms underlying this process are still largely unknown. In particular, the mechanisms how the Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc) directly drive reprogramming and which additional components are involved are still not yet understood. In this study, we aimed at analyzing the role of ADP-ribosyltransferase diphtheria toxin-like one (Artd1; formerly called poly(ADP-ribose) polymerase 1 [Parp1]) during reprogramming. We found that poly(ADP-ribosylation) (PARylation) of the reprogramming factor Sox2 by Artd1 plays an important role during the first days upon transduction with the reprogramming factors. A process that happens before Artd1 in conjunction with 10–11 translocation-2 (Tet2) mediates the histone modifications necessary for the establishment of an activated chromatin state at pluripotency loci (e.g., Nanog and Essrb) [Nature 2012;488:652–655]. Wild-type (WT) fibroblasts treated with an Artd1 inhibitor as well as fibroblasts deficient for Artd1 (Artd1−/−) show strongly decreased reprogramming capacity. Our data indicate that Artd1-mediated PARylation of Sox2 favors its binding to the fibroblast growth factor 4 (*Fgf4*) enhancer, thereby activating Fgf4 expression. The importance of Fgf4 during the first 4 days upon initiation of reprogramming was also highlighted by the observation that exogenous addition of Fgf4 was sufficient to restore the reprogramming capacity of Artd1−/− fibroblast to WT levels. In conclusion, our data clearly show that the interaction between Artd1 and Sox2 is crucial for the first steps of the reprogramming process and that early expression of Fgf4 (day 2 to day 4) is an essential component for the successful generation of iPSCs. STEM CELLS *2013;31:2364–2373*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The recent discovery of Takahashi and Yamanaka that it is possible to establish pluripotent stem cells, so called induced pluripotent stem cells (iPSCs), by reprogramming differentiated somatic cells [1] has opened new perspectives in the field of regenerative medicine. Therefore, many groups have worked on the refinement of reprogramming in order to optimize this technology for the use of iPSCs in clinical applications (reviewed in [2]). Nevertheless, the molecular mechanisms underlying the process of reprogramming are still largely unknown. An increased knowledge on how this process is driven and on the underlying mechanisms would lead to improved, more efficient reprogramming techniques.

Originally, reprogramming of mouse and human fibroblasts to iPSCs was performed by the retroviral-mediated introduction of the four transcription factors, Oct 4, Sox2, Klf4, and c-Myc (the so called Yamanaka factors [1]). How these factors directly drive the process of reprogramming and which additional components are involved still needs to be carefully analyzed. One of the Yamanaka factors, the transcription factor Sox2 (sex determining region Y-box 2), is a main player in maintaining pluripotency in embryonic stem cells (ESCs) [3]. Therefore, the regulation of Sox2 is most likely critical for the generation of iPSCs. One enzyme that has been demonstrated to post-translationally regulate Sox2 is the ADP-ribosyltransferase diphtheria toxin-like 1 (Artd1, formerly called Poly(ADP-ribose) polymerase 1/Parp1). Artd1 is a chromatin associated factor that catalyzes the covalent attachment of poly(ADP-ribose) (PAR) to itself and to other nuclear acceptor proteins [4,5]. ADP-ribosylation plays an important role in numerous biological processes, such as maintenance of genomic stability, cell differentiation, cell death, replication, and transcriptional regulation [6,7]. Different roles of Artd1 in the regulation and maintenance of pluripotency have also been previously described: deletion of Artd1 in ESCs globally affects gene expression patterns and

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Artd1 knockout ESCs differentiate into trophoblast derivatives [8,9]. Furthermore, it was previously shown that Artd1-dependent PARylation of Sox2 induces its eviction from the *Fgf4* (fibroblast growth factor 4) enhancer and thereby induces *Fgf4* transcriptional activation [10]. In contrast, recently Lai et al. reported that in ESCs, Artd1 PARylates itself and thereby enhances its interaction with Sox2, which in turn prevents Sox2 from binding to Oct4/Sox2 enhancers [11]. Previous work indicates an important role of Artd1 during reprogramming. Artd1 knockout (Artd1−/−) fibroblasts exhibit impaired reprogramming capacity [11], but the mechanisms underlying this observation were not analyzed. In a recent work, Doege et al. describe a role of Artd1 in conjunction with 10–11 translocation-2 (Tet2) in mediating the histone modifications necessary for the establishment of an activated chromatin state at pluripotency loci [12].

In this study, we aimed at analyzing the role of Artd1 during reprogramming, paying special attention to its role in the first days upon transduction (days 0–4) of the cells with the Yamanaka factors. We found that PARylation of Sox2 by Artd1 between day 0 and day 4 plays an important role in the generation of iPSCs. Inhibition of the enzymatic activity of Artd1 during this time period in wild-type (WT) fibroblasts resulted in a strongly decreased reprogramming efficiency after retroviral-mediated transduction of the Yamanaka factors. The same could be observed when using fibroblasts deficient for Artd1 (Artd1−/−). Our data further show that Artd1-mediated PARylation of Sox2 is involved in the regulation of *Fgf4* expression. The importance of Fgf4 during the first steps of reprogramming is also corroborated by our finding that addition of exogenous Fgf4 can rescue the reprogramming deficiency of the Artd1−/− cells.

In conclusion, our data clearly indicate a new role of Artd1 in regulating Fgf4 activity via Sox2 ADP-ribosylation during reprogramming and suggest a dual function of Artd1 during this process. Artd1 is essential for starting the Fgf4-mediated reprogramming process and later establishes the post-translation modification necessary for the activation of pluripotency genes [12].

Materials and Methods

Reprogramming

Mouse embryonic fibroblasts (MEF) were isolated from 14.5-day-pregnant C57BL/6 mice and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (PAA) and 1% L-glutamin/penicillin/streptomycin (10,000 U/ml penicillin G sodium; 10,000 μg/ml streptomycin sulfate; 29.2 mg/ml L-glutamine; 10 mM sodium citrate in 0.14% NaCl, Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com). The reprogramming of the MEFs was performed according to Yamanaka's protocol [13] using the pMXs retroviral vectors producing murine *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (Addgene, cat. nos. 13366, 13367, 13370, and 13375). Two days after infection, MEFs were cultured in DMEM containing 15% fetal bovine serum, 1% L-glutamin/penicillin/streptomycin, 1× MEM nonessential amino acids (Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com), and 50 mM ß-mercaptoethanol (Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com) supplemented with 1,000 U/ml ESGRO murine Leukemia inhibitory factor (Millipore, Chemikon, Zug, Switzerland, www.millipore.com). FGF4 (Sigma, Buchs, Switzerland, www.sigmaaldrich.com/switzerland-schweiz.html) was added during the reprogramming process at 10 ng/ml unless stated otherwise, ABT-888 (Enzo Life Sciences, New York, www.enzolifesciences.com) at 10 μM and SU5402 (Millipore, Calbiochem, Zug, Switzerland, www.millipore.com) at 2 μM.

Immunofluorescence Staining iPSCs

For immunofluorescence staining, iPSCs derived from WT, Artd1−/− fibroblasts, and Artd1−/− fibroblasts reprogrammed in the presence of Fgf4 (Artd1−/−*) were grown on mitomycin C-treated MEFs and fixed in 4% paraformaldehyde. Then, iPSCs were incubated with primary antibodies against Oct4 (rabbit anti-Oct4, Santa Cruz Biotechnology, Santa Cruz, CA, www.scbt.com) and SSEA-1 (mouse anti-SSEA-1, Millipore). Secondary fluorescence-labeled antibodies were used for detection (goat anti-rabbit Alexa Fluor 594 and goat anti-mouse Alexa Fluor 488, Molecular Probes, Invitrogen, Basel, Switzerland, www.invitrogen.com). Nuclei of the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Roche, Basel, Switzerland, www.roche.ch).

Real-Time PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Venlo, Netherlands, www.qiagen.com) and 1 μg of total RNA was reverse transcribed with Oligo-dT primers (Invitrogen) and Superscript III (Invitrogen). Real-time PCR was performed in triplicates in a Rotor-Gene Q RG-6000 (QIAGEN) with Rotor-Gene SYBR green (QIAGEN) and analyzed with the Delta Ct-method. GAPDH was used for normalization. Error bars represent the SD of the mean of triplicate reactions. Primers are listed in Supporting Information Table S1.

In Vitro Differentiation

For monoculture neural and smooth muscle differentiation, iPSCs (WT, Artd1−/− and Artd1−/− cells reprogrammed in the presence of Fgf4 [Artd1−/−*]) were plated onto gelatinized 35 mm dishes. The iPSCs were cultivated for 10 days with neural differentiation medium (DMEM/F-12 [Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com], N2 [1:100, Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com], B27 [1:50, Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com], and 1% L-glutamin/penicillin/streptomycin) or smooth muscle differentiation medium (DMEM and 10% fetal bovine serum). At day 10, cells were fixed in 4% paraformaldehyde and stained for βIII-tubulin (Sigma) and smooth muscle actin (Sigma), respectively.

Western Blotting

Cells were collected in radioimmunoprecipitation RIPA buffer (50 mM Tris-HCl pH 8; 400 mM NaCl; 0.5% Nonidet P40; 1% Na-Deoxycholate; 0.1% SDS; Protease Inhibitor Cocktail Tablet, EDTA-free Roche, IN). Proteins were identified by SDS-PAGE (10% acrylamide) and Western blotting using the following antibodies: α-PAR ALX-210–890 (Enzo Life Sciences, New York, www.enzolifesciences.com), α-PARP-1/2 (H250) sc-7150, α-Sox2 15830 (Abcam, Cambridge, United Kingdom, www.abcam.com), α-Pcna (PC10) sc-56, α-tubulin T6199 (Sigma), IRDye 800CW anti-Rabbit, and IRDye 680RD anti-Mouse (LI-COR, Lincoln, Nebraska, www.licor.com). Images were acquired with an Odyssey Imaging System (LI-COR).

In Vitro Sox2 ADP-Ribosylation

HEK293 cells were seeded at a density of 2.7×10^6/150 mm dish and after overnight incubation transfected with pBluescript II and pCAG-HA-Sox2-IP (cat. no. 13459) vectors, respectively (using $CaCl_2$ transfection). After 72 hours, cells were harvested and resuspended in NE buffer (50 mM Tris-HCl [pH 7.5], 0.15 M KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 20% [vol/vol] Glycerol). After sonication at 4°C for 2 × 30-second, the cells were incubated with DNase (Fermentas, Thermo Fisher Scientific Waltham, Massachusetts, http://www.thermoscientificbio.com) for 30 minutes at 4°C. After DNA digestion, the cells were sonicated for 30 seconds and then centrifuged at 6,000 rpm for 10 minutes. The cleared lysate was subjected to immunoprecipitation overnight at 4°C using immobilized antibody against HA (ANTI-HA affinity gel, Sigma). Precipitates were washed three times with

NE buffer and after centrifugation; the HA-Sox2 coupled beads were resuspended in reaction buffer (50 mM Tris-HCl pH8, 4 mM $MgCl_2$, 0.25 mM dithiothreitol, 5 mM NaCl, 200 nM EcoRI linker). Recombinant human ARTD1 (10 pmol) and ^{32}P-NAD+ (4 nmol) were added and the reactions were incubated for 15 minutes at 30°C. Empty pBlueScript II vector was used as a negative control and 0.1 μg of histone H1 (10223549001; Roche) as a positive control. Proteins were resolved by SDS-PAGE (10% acrylamide), exposed to x-ray film (Tx-RP) and analyzed (Typhoon imager, GE Healthcare Life Sciences, Switzerland, http://www.gelifesciences.com).

High Stringency Immunoprecipitation

At day 4, cells were collected in cold phosphate buffered saline and lysed in hypotonic buffer (5 mM Hepes pH 7.5; 85 mM KCl; 0.5% Nonidet P40; protease inhibitor Roche), and nuclei were spun down for 10 minutes at 8,000 rpm. Nuclei were then suspended in High Stringency Buffer (50 mM Tris-HCl pH 7.5; 0.4 M NaCl; 1% Nonidet P40; 0.4% Na-Dedoxycholate) followed by sonication and DNA digestion with DNaseI (Roche). Extracts were cleared by 10 minutes centrifugation at 14,000 rpm. Cleared nuclear extracts were diluted 1:2.7 in 50 mM Tris-HCl pH 7.5 and immunoprecipitations were carried out for 2 hours using 10H antibody or IgG as a negative control. Beads were then washed three times in the same buffer and lastly boiled in SDS loading buffer.

Immunofluorescence Staining During Reprogramming

WT and Artd1−/− fibroblasts were seeded on glass coverslips and reprogramming was induced as previously described. At the indicated time, cells were fixed in acetic acid/methanol (1:3) for 5 minutes on ice, blocked for 30 minutes in PBSMT (phosphate buffered saline containing 5% milk and 0.05% Tween-20), incubated with α-Sox2 (15830, Abcam, 1:200) and α-PAR (10H, Enzo Life Sciences, New York, www.enzolifesciences.com) (1:250) dissolved in PBSMT for 1 hour at room temperature, washed with phosphate buffered saline, incubated with secondary Alexa Fluor 488 α-rabbit (Invitrogen) and Cy3-IgG fraction monoclonal mouse anti-FITC antibodies, and embedded on microscopy slides with DAPI containing mounting medium VECTASHIELD. Images were acquired with a Leica SP5 microscope at the Centre for Microscopy and Image Analysis of the University of Zürich.

Sox2 Coimmunoprecipitation

WT and Artd1−/− fibroblasts were reprogrammed as previously described. At the indicated time, cells were harvested and lysed in hypotonic buffer (5 mM Hepes, 85 mM KCl, 0.5% Nonidet P40, Protease Inhibitor [Roche]). Nuclei were pelleted at 8,000 rpm for 10 minutes at 4°C and resuspended in immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, protease inhibitor [Roche], 0.5 mM dithiothreitol), sonicated, and DNA was digested with DNaseI (Roche). Immunoprecipitations were carried out using 200 μg of nuclear proteins and 2 μg of α-Sox2 (15830, Abcam) for 2 hours at 4°C, followed by three washes in washing buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.1% Tween-20, protease inhibitor [Roche]), and eventually resuspended in 1× SDS-loading buffer.

Chromatin Immunoprecipitation

WT, Artd1−/−, and ABT-888 inhibited cells were crosslinked with 1% formaldehyde (Calbiochem). Chromatin was fragmented with the Bioruptor (Diagenode, Liège, Belgium, http://www.diagenode.com), incubated with specific antibodies, and collected with Protein A Agarose/salmon sperm DNA (Millipore). DNA was extracted and measured by real-time PCR using SYBR Green and Rotor-Gene 3000 (Corbett Life Science/QIAGEN). For Primer sequences see Supporting Information Table S1.

RESULTS

Artd1 Is Necessary for Successful Initiation of Reprogramming

MEFs were isolated from day 14 embryos obtained from homozygous breeding of WT and Artd1−/− mice, respectively. WT and Artd1−/− fibroblast were transduced with the Yamanaka factors as previously described [13] and the number of iPSC-colonies was assessed after 14 days of cultivation. The number of iPSC colonies obtained upon reprogramming of Artd1−/− fibroblasts was reduced by around 65% compared to WT fibroblasts (Fig. 1A). These observations are in agreement with recently published data [11] and indicate that Artd1 is required for the reprogramming of somatic cells to iPSCs. An even stronger reduction in the number of iPSC colonies (80%) was observed when the PARP-Inhibitor ABT-888, which mainly inhibits Artd1 and Artd2 [14], was applied. The small difference in the number of colonies between cells lacking Artd1 and PARP-inhibitor treated cells might indicate that the contribution of other ARTD family members than Artd1 during reprogramming is minimal. In addition, the ABT-888 inhibitor did not change the transcriptional levels of Artd1 and Artd2, indicating that the effects observed are exclusively due to the inhibition of the enzymatic activity (Supporting Information Fig. S1A, S1B). In order to define if ADP-ribosylation is necessary during the whole reprogramming process or only during a specific time window, we added the ABT-888 inhibitor starting from days 0, 2, 4, 6, and 8 after the transduction of WT fibroblasts with the Yamanaka factors. iPSC colony formation was strongly reduced when the inhibitor was added during the first 4 days of reprogramming, but unaffected if cells from later time points after viral infection were treated (Fig. 1B), indicating that Artd1 enzymatic activity is essential during the early phase of the reprogramming process. In order to determine the expression changes of Artd1 and Artd2 during the first 12 days of reprogramming, we performed quantitative real-time PCR analysis (Fig. 1C, 1D). The expression of both Artd1 and Artd2 constantly increased from day 2 to day 8 and dropped to levels similar to the untreated control cells by day 12. This increase in Artd1 expression was also observed at the protein level (Fig. 1E) and was in accordance with increased PARylation in fibroblasts at day 2, followed by a decrease at day 4 and a constant level in the following days (Fig. 1F). In summary, our data indicate an upregulation of Artd1 and Artd2 expression and of PARylation during the first days of reprogramming. Blocking of Artd1, either by genetic ablation or by applying a PARP-inhibitor, drastically decreases the efficiency of reprogramming.

Artd1 Is Responsible for Poly(ADP-Ribosylation) of Sox2 During Reprogramming

In ESCs, Artd1 was previously described to PARylate Sox2, thereby decreasing the association of Sox2 with the *Fgf4* enhancer and inducing Fgf4 expression [10]. In order to test the capacity of Artd1 to PARylate Sox2, we transfected a HA-Sox2 expression vector in HEK293 cells and the recombinant protein was purified by immunoprecipitation. Upon in vitro incubation of the purified protein with recombinant human ARTD1 and radiolabeled NAD^+, a signal at the predicted size of Sox2 was clearly detected, indicating that Sox2 is substrate of ARTD1 (Fig. 2A). To further prove that Sox2 is PARylated during reprogramming, WT, WT+ABT-888, and Artd1−/− day 4 cells were collected in high stringency ionic buffer in order to reduce cellular protein complexes. Cleared nuclear lysates were subsequently used to immunoprecipitate PARylated proteins with an anti-PAR antibody. PARylated

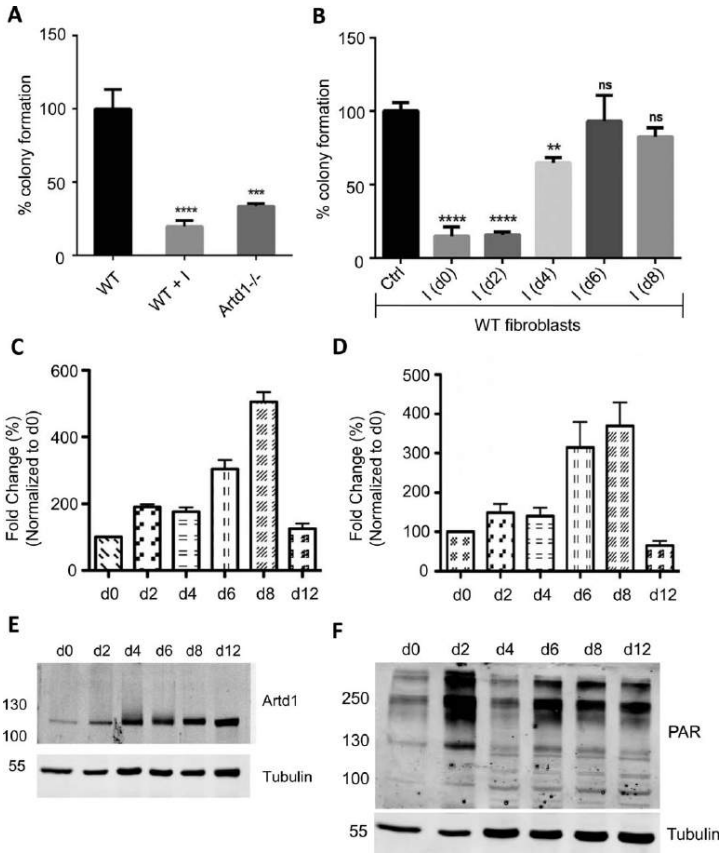

Figure 1. Artd1 enzymatic activity is necessary for the initial steps of reprogramming. **(A):** Reprogramming efficiency in WT fibroblasts, Artd1−/− fibroblasts, and WT fibroblasts treated with ABT-888. **(B):** Artd1 enzymatic activity is essential during the early phase of the reprogramming process. ABT-888 was added at the indicated time points and the reprogramming efficiency was assessed. **(C):** Expression of Artd1 in WT cells during the reprogramming process. **(D):** Expression of Artd2 in WT cells during the reprogramming process. **(E):** Expression levels of Artd1 during reprogramming. Western blot for Artd1 on WT cells collected at the indicated time points during reprogramming. The smear of the Artd1 signal reflects Artd1 activity. Tubulin has been used as a loading control. Molecular size references in kilo Daltons are indicated. **(F):** PAR formation during reprogramming. Western blot for PAR on WT cells collected at the indicated time during reprogramming. Tubulin has been used as a loading control. Molecular size references in kilo Daltons are indicated. Dunnett's multiple comparison test. ns = not significant, **, $p < .01$; ***, $p < .001$; ****, $p < .0001$. Abbreviations: PAR, poly(ADP-ribose); WT, wild type.

proteins were resolved in SDS-PAGE, followed by Western blotting against Sox2 and Artd1. Sox2 as well as Artd1 were pulled down in WT extract but not in WT+ABT-888 or Artd1−/− extracts, indicating that Sox2 is a targeted by ADP-ribosylation in vivo. (Fig. 2B) In order to assess the expression pattern of Sox2 and the PARylation levels during the first phase of reprogramming, we performed Western blotting of Sox2 (Supporting Information Fig. S2) and monitored the localization of Sox2 and PAR by immunofluorescence in WT and Artd1−/− fibroblast at days 0, 2, 4, and 6 after viral infection with the Yamanaka factors (Fig. 2C and Supporting Information Fig. S3). In the nuclei of WT fibroblasts PARylation was detectable starting from day 2 post-transduction and was still present at day 6. Interestingly, Sox2 staining mainly colocalized with the PAR signal, suggesting that ADP-ribosylation of Sox2 occurs at the beginning of reprogramming. As expected, a PAR signal was not detectable in the Artd1−/− fibroblasts at any time point, thus supporting the idea that Artd1 is the major ADP-ribosyltransferase involved in the process.

In order to clarify if in vivo, during the first phases of reprogramming, Artd1 and Sox2 directly interact, WT and Artd1−/− fibroblasts were transduced with the Yamanaka

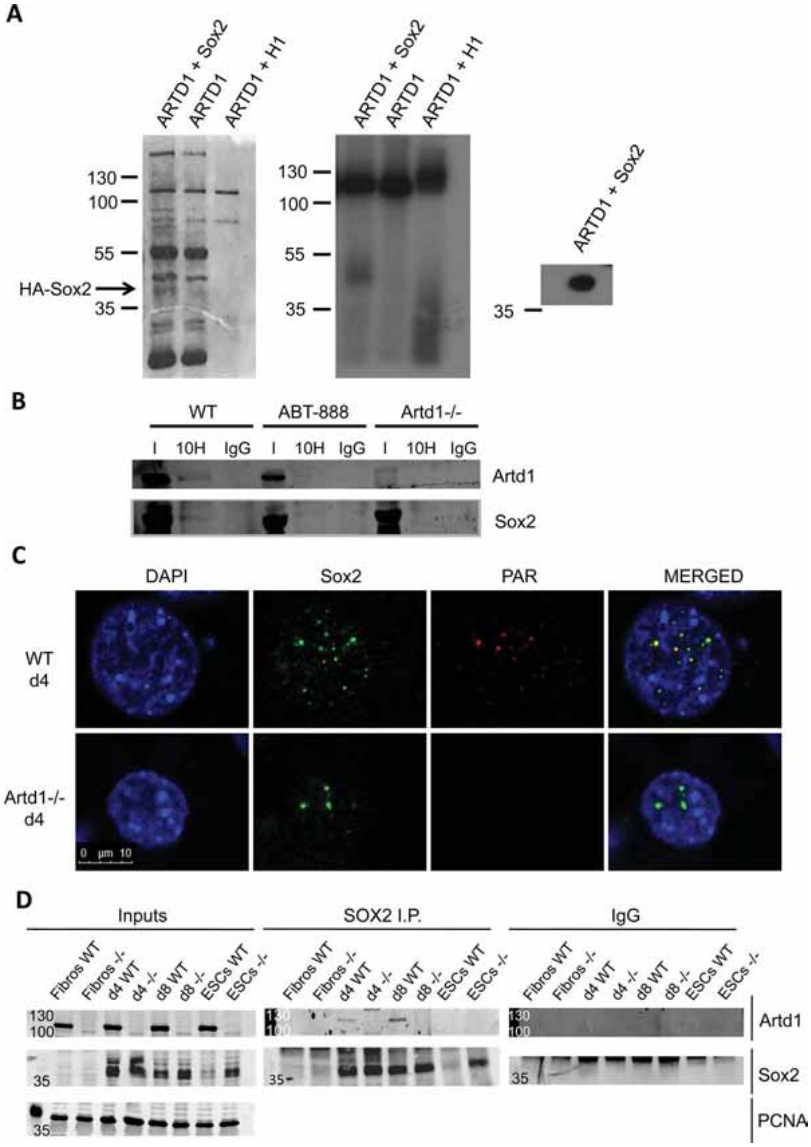

Figure 2. Artd1 binds and post-translationally modifies Sox2 during reprogramming. **(A):** Trans ADP-ribosylation of HA-Sox2 by recARTD1. Recombinant human ARTD1 (10 pmol) was incubated with HA-Sox2 (lane 1), HA-empty (lane 2), or H1 as positive control (lane 3). Coomassie blue stained gel (left), autoradiography (middle), and Western blot for Sox2 bound to the beads (right) are shown. **(B):** Sox2 ADP-ribosylation during reprogramming. High stringent immunoprecipitation was carried on with either PAR antibody 10H or IgG control on nuclear extracts of WT, ABT-888 treated WT, and Artd1−/− cells collected at day 4. Western blots of Sox2 and Artd1 in immunoprecipitation samples and inputs are shown. **(C):** Immunofluorescence of Sox2 and PAR during reprogramming at day 4. WT and Artd1−/− cells were stained with DAPI, for Sox2 and for PAR. Signals in the relative channels are shown from left to right. Merge of the three channels is also reported. Scale bar = 10 µm. **(D):** Sox2/Artd1 interaction during reprogramming. Immunoprecipitation was carried on with either Sox2 antibody or IgG control on nuclear extracts of WT and Artd1−/− cells collected at the indicated time. Western blots of Sox2 and Artd1 in immunoprecipitation samples and inputs are shown. proliferating cell nuclear antigen (PCNA) was used as indicator of equal starting protein content for the immunoprecipitations. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ESCs, embryonic stem cells; Fibros, fibroblasts; WT, wild type.

factors and protein extracts were isolated at day 4 and day 8 upon transduction. Artd1/Sox2 coimmunoprecipitation was detectable at day 4 and day 8 in WT fibroblasts, clearly indicating that these two proteins interact in reprogramming fibroblasts. As expected, in Artd1−/− fibroblasts, no interaction was detectable (Fig. 2D). Taken together, our data show that within the first 8 days of reprogramming, Artd1 interacts with Sox2 and mediates its PARylation.

Artd1-Mediated ADP-Ribosylation of Sox2 Is Responsible for the Activation of Fgf4 Transcription

Although Sox2 has been reported to bind to the *Fgf4* enhancer element, its effect on *Fgf4* transcription remains controversial. Originally, it was described that Sox2 binding enhances *Fgf4* transcription [15]. In contrast, a more recent study reported that in ESCs and in differentiating cells, Sox2 represses *Fgf4* transcription and that ADP-ribosylation of Sox2 relives Fgf4 repression [10]. In addition, the work of Lai et al. showed that in ESCs, Sox2 positively regulates *Fgf4* transcription and that auto-modified Artd1 interacts with Sox2, consequently inducing its release from the *Fgf4* enhancer and repressing *Fgf4* transcription [11]. To investigate the role of Sox2, Artd1, and ADP-ribosylation in the fine-tuning of *Fgf4* transcription, we first analyzed the expression of *Fgf4* upon initiation of reprogramming. In WT fibroblasts, expression of *Fgf4* was detectable starting from day 2 and increased steadily until day 6. In contrast, in Artd1−/− as well as in WT fibroblasts treated with the ABT-888 inhibitor, expression was strongly reduced and delayed. This clearly indicates that the presence of Artd1 is necessary for the correct activation of *Fgf4* transcription (Fig. 3A). In order to assess the binding capacity of Sox2 to the *Fgf4* enhancer, we performed ChIPs using antibodies against Sox2 during reprogramming in WT, Artd1−/−, and in WT fibroblasts treated with ABT-888. As depicted in Figure 3B, during the reprogramming process, Sox2 is recruited to the *Fgf4* enhancer and to other target sites such as the *Nanog* promoter. In ABT-888 inhibited fibroblasts, Sox2 recruitment is delayed, which is in agreement with the lower transcription of *Fgf4* (Fig. 3A), indicating that PARylation positively influences the DNA binding capacity of Sox2. Strikingly, the delay of Sox2 recruitment in the presence of ABT-888 is phenocopied in Artd1−/− cells (Fig. 3B), confirming that Artd1 is mainly responsible for Sox2 ADP-ribosylation in reprogramming cells. Taken together, our data demonstrate that ADP-ribosylation of Sox2 strengthens the binding of Sox2 to its target sites and thereby stimulates the transcription of the corresponding target genes.

Fgf4 Expression Is Crucial for the Initiation of Reprogramming

The reduced transcription of *Fgf4* in Artd1−/− fibroblasts upon the initiation of reprogramming leads to the question if the impaired reprogramming efficiency of Artd1−/− cells is the direct consequence of the reduced Fgf4 levels or if other factors are involved. We therefore repeated the reprogramming experiments in WT and Artd1−/− fibroblast by adding 10 ng/ml and 25 ng/ml of exogenous Fgf4 to the cells. Addition of 10 ng/ml of Fgf4 was sufficient to restore the reprogramming efficiency to comparable levels as in WT cells (Fig. 4A), indicating that the phenotype observed in Artd1−/− cells is due to an insufficient expression of Fgf4 during the early stages of reprogramming. Interestingly, higher amounts of Fgf4 (25 ng/ml) or addition of exogenous Fgf4 to WT fibroblast rather impaired the reprogramming efficiency, suggesting that a tight control of autocrine Fgf4 production is essential for the initiation of reprogramming. The Artd1−/− iPSC colonies obtained upon addition of Fgf4 could be expanded for more than 10 passages and expressed the classic pluripotency genes. Furthermore, they were able to differentiate in vitro toward smooth muscles and neurons and showed no differences from WT and Artd1−/− iPSCs (Supporting Information Fig. S4).

The fact that Fgf4 transcription depends on ADP-ribosylation of Sox2 and the capacity of exogenous Fgf4 to restore the reprogramming efficiency in Artd1−/− fibroblasts suggests that the enzymatic activity of Artd1 is essential for the initiation of reprogramming. To test this, we reprogrammed WT fibroblasts and cultivated the cells for the first 2, 4, or 6 days with ABT-888 or with ABT-888 and 10 ng/ml Fgf4. The addition of Fgf4 for the first 2 or 4 days abolished the inhibitor effect of ABT-888 and significantly increased the reprogramming efficiency (Fig. 4B), indicating that the direct addition of Fgf4 compensates for the absence of ADP-ribosylation activity. This strengthens the observation that Artd1-mediated ADP-ribosylation of Sox2 is essential to modulate Fgf4 transcription during the initial phases of reprogramming. To further prove the importance of Fgf4, we performed Fgf4 knockdown experiments. The combination of viral transduction of the reprogramming factors with knockdown induced massive cell death in the fibroblast. This was not due to Fgf4 knockdown itself because scrambled controls had the same effect. We therefore decided to inhibit Fgf receptor tyrosine kinases with small chemical inhibitor SU5402 [16] in WT cells. The inhibition of Fgf receptor tyrosine kinases reduced the reprogramming efficiency of WT fibroblasts by around 50% (Fig. 4C). In summary, our results clearly identify Artd1-mediated ADP-ribosylation of Sox2 as an essential component for the correct activation of Fgf4 expression, which in turn plays a crucial role for the initiation of the reprogramming process.

Discussion

Cell differentiation is normally an irreversible process and differentiated cells are not able to switch from one lineage to another. Cellular reprogramming to pluripotency therefore requires that pluripotency genes, which are inactive in differentiated cells, are reactivated. Exogenously introduced reprogramming factors must therefore bind and reactivate their target genes in tight collaboration with other endogenous factors, particularly epigenetic regulators.

In this work, we aimed at understanding the function of the well-known epigenetic regulator Artd1 during the first phase of reprogramming. Artd1 was previously shown to be necessary for proper differentiation of ESCs [8]. Even though Artd1 deficiency does not affect the growth of ESCs, its absence compromises cell survival and growth when ESCs are induced to differentiate [8,10]. The molecular mechanisms underlying this observation are still largely unclear, but new studies indicate that Artd1 acts as a cofactor of Oct4 and Sox2 in ESCs by binding to the *Fgf4* enhancer and thereby regulates Fgf4 expression [10,11]. The major function of Fgf4 in pluripotent cells is to regulate the selection between the alternative fates of self-replication and lineage commitment during continuous proliferation. Autocrine production of Fgf4 is the major stimulus activating the Erk1/2 signaling cascade in naïve mouse ESCs [17,18]. Inhibiting ERK and FGF activity with small chemical compounds prevents ESCs from differentiating without affecting the propagation of the

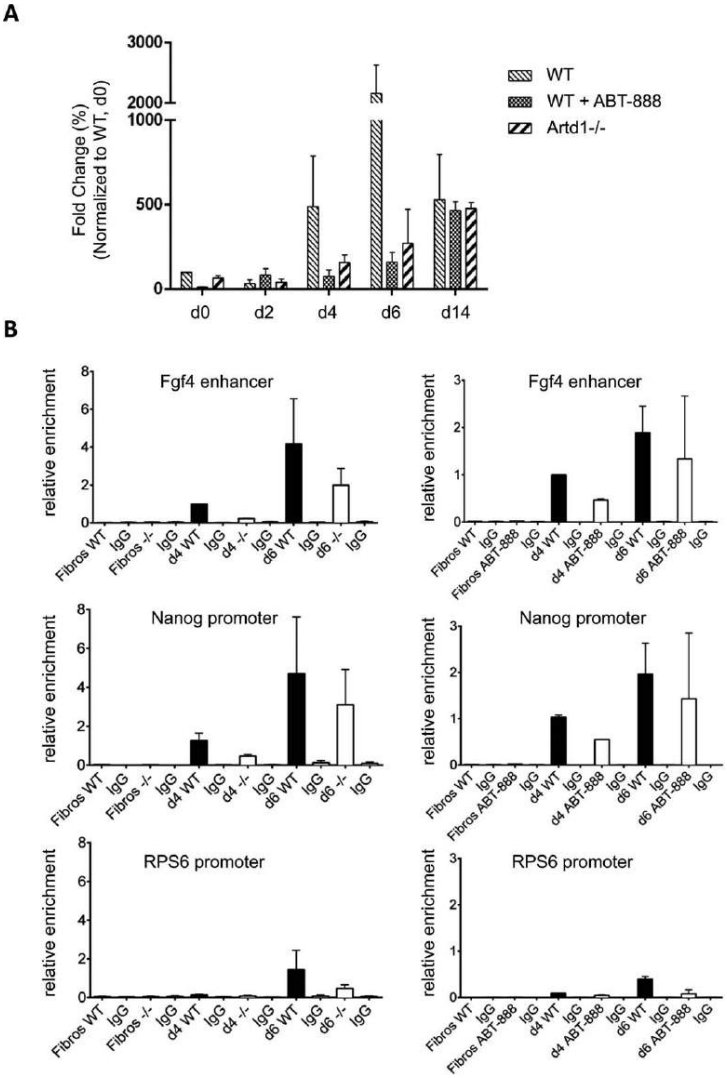

Figure 3. Artd1 activity is necessary for the binding of Sox2 to the Fgf4 enhancer and for driving Fgf4 expression. **(A):** Expression of Fgf4 during the reprogramming process in WT fibroblasts ± ABT-888 and Artd1−/− fibroblasts. **(B):** Recruitment of Sox2 to target genes: chromatin immunoprecipitation was carried on with either Sox2 antibody or IgG control in WT, Artd1−/−, and ABT-888 inhibited cells at the indicated time points. Recruitment of Sox2 to the Fgf4 enhancer, the Nanog promoter, and the unrelated Rps6 promoter is depicted. Values are expressed as enrichment over input signals. Abbreviation: WT, wild type.

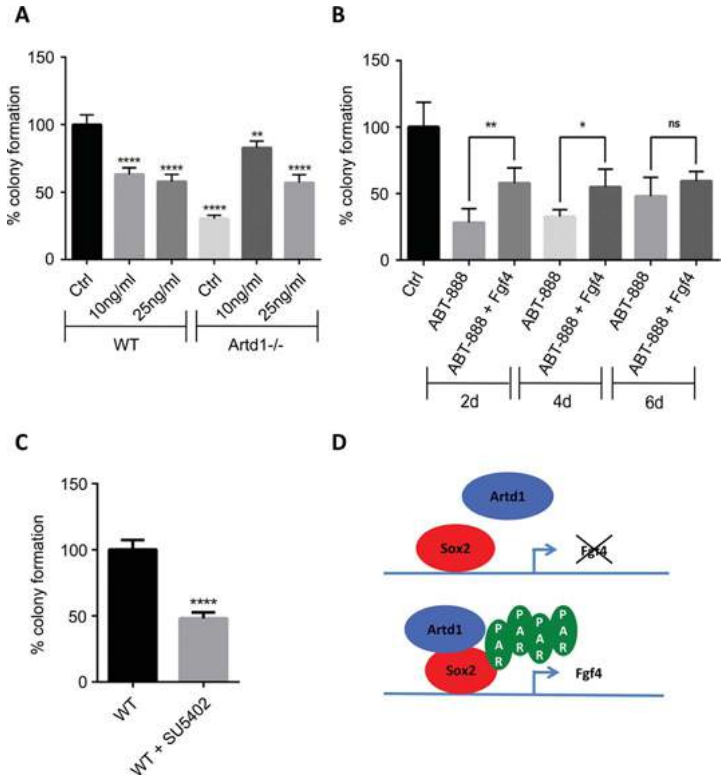

Figure 4. Exogenous Fgf4 supplementation is sufficient to restore the reprogramming capacity in Artd1−/− fibroblasts and WT cells treated with ABT-888 inhibitor. **(A):** Reprogramming efficiency of Artd1−/− cells cultivated with exogenously added Fgf4. **(B):** Reprogramming efficiency of WT+ABT-888 cells in the presence or absence of Fgf4. WT fibroblasts were treated for the first 2, 4, or 6 days with ABT-888 alone or ABT-888 and 10 ng/ml Fgf4. **(C):** Reprogramming efficiency in WT cells and WT cells treated with SU5402, an inhibitor of Fgf receptor tyrosine kinase activity. **(D):** Schematic representation of Artd1-mediated PARylation of Sox2 and binding to the Fgf4 enhancer, which activates Fgf4 expression. Dunnett's multiple comparison test (A) and Student's *t* test (B, C). ns = not significant, *, $p > .05$; **, $p < .01$; ****, $p < .0001$. Abbreviation: WT, wild type.

undifferentiated ESCs [18,19]. Similarly, ESCs lacking Fgf4 are resistant to neural and mesodermal induction, but are able to commit when FGF is provided exogenously [18].

During differentiation of ESCs, Artd1 was shown to directly interact with and to PARylate Sox2, leading to the dissociation and degradation of Sox2 from the *Fgf4* enhancer. This releases Sox2 inhibition and induces *Fgf4* gene transcription [10]. In the absence of activated Artd1, Sox2 cannot be ADP-ribosylated, augmenting its interaction with the *Fgf4* enhancer and leading to a stabilization of Sox2 protein and a reduction in Fgf4 levels [10]. An alternative model suggests that Artd1 auto-PARylation enhances Sox2-Artd1 interactions and inhibits binding of Sox2 to the Oct4/Sox2 site at the *Fgf4* enhancer. This process seems to be regulated by FGF/ERK signaling [11].

We first tested the reprogramming capacity of Artd1 knockout cells and found that the absence of Artd1 strongly reduces the number of iPSC colonies. We could also identify that the critical time period during which Artd1 activity is necessary are the first 2–4 days after transduction with the reprogramming factors. This is in agreement with the increased expression of Artd1 starting at day 2 after reprogramming and the concomitant increase of PARylation. The reduction of the reprogramming efficiency is mainly due to the lack of Artd1 activity and not of other members of the ARTD family, because the treatment of WT fibroblasts with ABT888, an inhibitor of poly(ADP-ribosyltransferases), mimics the genetic ablation of Artd1. We further observed a strong delay in *Fgf4* expression upon the initiation of reprogramming in Artd1−/− fibroblasts or when WT fibroblasts are treated with ABT-888. Fgf4 expression occurs much earlier than the activation of transcription of other typical pluripotency markers such as Nanog, SSEA-1, or OCT-4. This observation is interesting because Fgf4 is typically expressed in pluripotent cells [18,20,21] and not in fibroblasts.

Based on previous studies [10,11] indicating a role of Artd1 in modulating Sox2 activity in the context of Fgf4 regulation, we decided to analyze the capacity of Artd1 to modify Sox2 in vitro and in vivo. Our data demonstrate that human ARTD1 is able to PARylate Sox2 in vitro and strongly suggest that murine Artd1 mediates ADP-ribosylation of Sox2 in vivo in fibroblasts starting from day 2 during reprogramming. The role of Sox2 in the regulation of *Fgf4* transcription is also highlighted by the observation that ADP-ribosylation of Sox2 increases its binding to the *Fgf4* enhancer and leads to increased transcription. In summary, our data confirm that during the early phase of the reprogramming process, Artd1-mediated ADP-ribosylation of Sox2 is necessary for the binding of Sox2 to the *Fgf4* enhancer and for inducing Fgf4 expression, which in turn is responsible for initiating the further events leading to the formation of iPSCs.

The importance of Fgf4 during the first phase of the reprogramming process is strengthened by the fact that Artd1−/− fibroblasts, which show a strongly reduced activation of *Fgf4* upon reprogramming initiation, show a massive reduction in the number of iPSC colonies. The simple addition of Fgf4 during this time is sufficient to restore the reprogramming efficiency to comparable levels as in WT cells, indicating that Fgf4 is functionally the only factor regulated by Artd1 during the early phase of reprogramming (Fig. 4A). A similar effect can be observed when WT fibroblasts are cultivated in the presence of the ABT-888 inhibitor. Also in this case the simple addition of Fgf4 to the medium is sufficient to restore the reprogramming efficiency (Fig. 4B). The importance of Fgf4 during the early phase of reprogramming is also corroborated by the fact that WT cells treated with an inhibitor of Fgf receptor tyrosine kinases, which are normally activated upon the binding of Fgf, reduces the reprogramming efficiency by 50% (Fig. 4C).

Of interest, Artd1 in conjunction with 10–11 translocation-2 (Tet2) was recently shown to play an important role in the early stages of somatic cell reprogramming by mediating the histone modifications necessary for the establishment of an activated chromatin state at pluripotency loci (e.g., Nanog and Essrb) [12]. Furthermore, Artd1 induction promotes accessibility to the Oct4 reprogramming factor. Interestingly, pluripotency factors are detectable starting around days 10–12 of the reprogramming process [22–25], which would suggest that Artd1 might have two different functions. In the first phase (first week) of the reprogramming process, Artd1 is required for initiating the transcription of Fgf4. In a second step, Artd1 might be involved in promoting the accessibility of the reprogramming factors to the pluripotency gene promoters as shown by Doege et al. [12]. Since we did not observe an effect of PARP inhibitors on the reprogramming efficiency at this time point, it is fair to assume that this process is independent of ADP-ribosylation. Furthermore, our data demonstrate that even in the absence of Artd1, exogenously supplied Fgf4 permits reprogramming efficiencies as for WT fibroblasts, indicating that in the ARDT1−/− cells additional epigenetic modifiers must be interacting with Tet2 to mediate the histone modifications necessary for the activation of pluripotency genes.

Conclusions

Our data indicate that PARylation of Sox2 by Artd1 plays an important role in the generation of iPSCs. Artd1-mediated PARylation of Sox2 favors its binding to the Fgf4 enhancer, thereby activating *Fgf4* expression (Fig. 4D). Exogenous addition of Fgf4 during the first 4 days upon initiation of reprogramming was sufficient to restore the reprogramming capacity of Artd1 knockout fibroblast to WT levels, indicating that Fgf4 is an essential component for the correct initiation of the reprogramming process.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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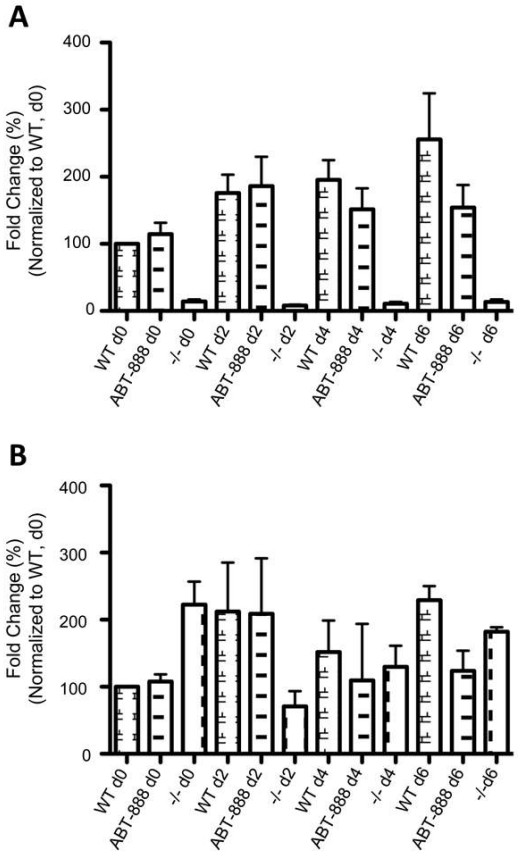
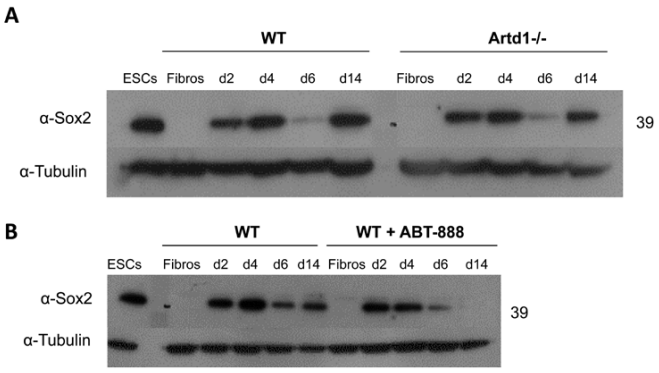
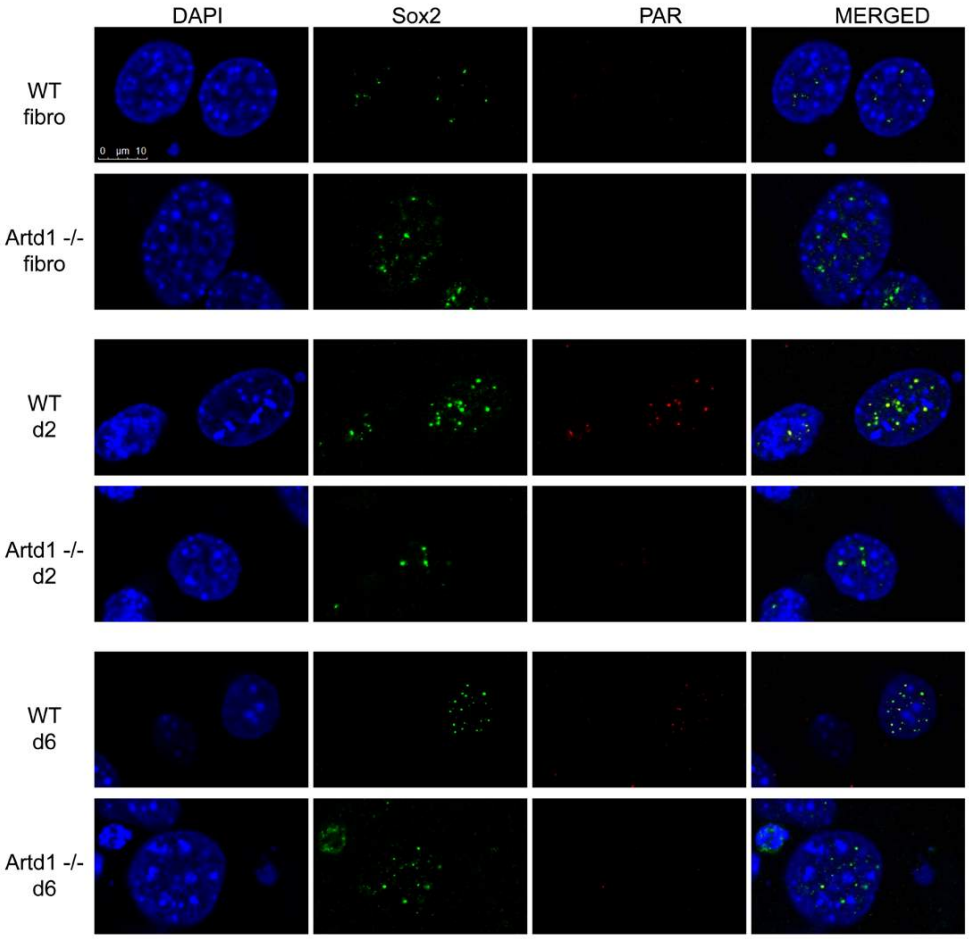


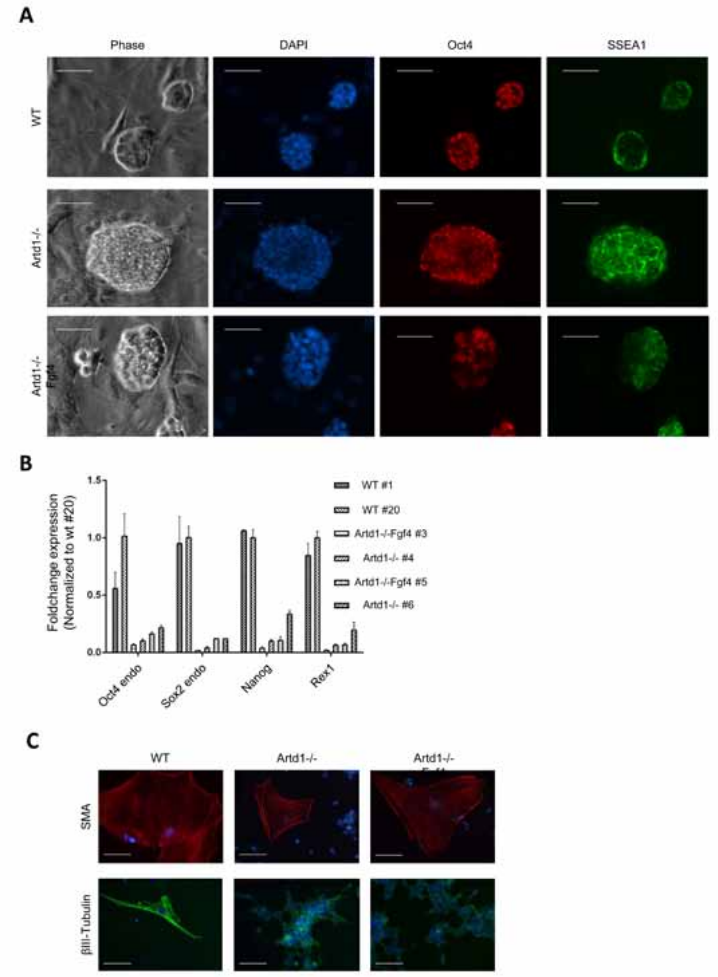
Supplementary Figure 1. PARP-inhibitor do not change the transcriptional levels of Artd1 or Artd2. **(A):** Artd1 expression in wild-type (wt) cells, PARP-inhibited (ABT-888) cells and Artd1 knock-out cells **(B):** Artd2 expression in wild-type (wt) cells, PARP-inhibited (ABT-888) cells and Artd1 knock-out cells Artd1



Supplementary Figure 2. Artd1 knock out or inhibition of ADP-ribosylation do not change Sox2 protein levels. (A): Sox2 western blot in reprogramming wild-type (wt) or Artd1 knock-out (Artd1-/-) fibroblasts. (B): Sox2 western blot in reprogramming wild-type (wt) or PARP-inhibited (ABT-888) fibroblasts. Tubulin was used as loading control.



Supplementary Figure 3. Artd1 is necessary for the reprogramming-induced PAR formation. **(A):** Sox2 and PAR immunofluorescence at different time points during reprogramming. DAPI was used as nuclear marker.



Supplementary Figure 4. Reprogrammed ARTD1-/- fibroblasts with exogenous Fgf4 administration are pluripotent and able to differentiate to smooth muscles and neurons. **(A):** Oct4 and SSEA1 immunofluorescence of wild-type (wt), Artd1 knock out (Artd1-/-) and Artd1 knock out with exogenous administration of Fgf4 (Artd1-/- Fgf4) iPS cells **(B):** Oct4, Sox2, Nanog and Rex1 expression in wild-type (wt), Artd1 knock out (Artd1-/-) and Artd1 knock out with exogenous administration of Fgf4 (Artd1-/- Fgf4) **(C):** SMA and βIII-tubulin immunofluorescence of wild-type (wt), Artd1 knock out (Artd1-/-) and Artd1 knock out with exogenous administration of Fgf4 (Artd1-/- Fgf4) iPS cells

3.2 Submitted manuscript

A new highly specific ChAP method for the genome-wide localization of chromatin ADP-ribosylation

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Abstract

Chromatin ADP-ribosylation is catalyzed by diphtheria toxin-like ADP-ribosyltransferases (ARTDs). However, the lack of suitable methods has so far prevented genome-wide analysis of chromatin ADP-ribosylation. We have therefore developed a novel chromatin affinity precipitation (ChAP) technique based on the affinity of two poly-ADP-ribose binding domains (WWE of RNF146 and macrodomain Af1521) to map poly-ADP-ribosylation of chromatin regions at the genome-wide scale. ChAP performed on oxidatively stressed A549 epithelial cells revealed that chromatin ADP-ribosylation is associated with SINE and LINE, as well as alpha satellite repetitive elements. Interestingly, ADP-ribsylation was associated with hetero- rather than euchromatin and with enhanced accessibility to the DNA. Moreover, application of the ChAP in differentiating adipocytes revealed that chromatin ADP-ribosylation predominantly localizes at promoters of PPARγ target genes. Together, this method allows investigating how ADP-ribosylation regulates chromatin plasticity and functions during biologically important processes.

Introduction

Protein ADP-ribosylation is an ancient and reversible post-translational protein modification with high biochemical complexity (linear and/or branched) that comprises the transfer of the ADP-ribose moiety from NAD^+ to specific amino acid residues on substrate proteins or to ADP-ribose itself, generating protein poly-ADP-ribosylation. ADP-ribosylation is primarily governed by ADP-ribosyltransferases (22 mammalian enzymes identified so far), recognized by different protein domains and thus also serve as a scaffold for the recruitment of proteins during complex formation ('readers'), and de-modification by ADP-ribosylhydrolases ('erasers'). Among the described readers, some specifically recognized mono-ribosylated (MARylated) and poly-ADP-ribosylated (PARylated) proteins (e.g. macrodomain Af1521), while other bind only PARylated proteins (e.g. WWE domain). MARylation or PARylation alters the function of modified proteins or provides a scaffold for the recruitment of other proteins and thus regulates several cellular processes[51]. From the existing 18 intracellular ARTDs (ART diphteria toxin-like), ARTD1 is found exclusively in the nucleus while the other ARTDs are found in the nuclear as well as in the cytoplasmic or only in the cytoplasmic compartment. Interestingly, most if not all poly-ADP-ribose (PAR) chains are found on nuclear proteins[127]. In general, the currently detectable nuclear PAR formation mainly seems to be induced as a response to stress signaling, and the intensity of ADP-ribosylation seems to correlate with the intensity of the signal[128].

Cellular studies have shown that ARTD1 is recruited to eu- as well as heterochromatin. The earliest studies have focused on the activation of ARTD1 enzymatic activity by DNA lesions induced by exogenous stimuli such as hydrogen peroxide (H_2O_2). H_2O_2 treatment is assumed to introduce DNA lesions randomly especially in open euchromatin[129]. In contrast, there are also recent indications that nuclear PARylation by ARTD1 is induced upon a very targeted process, e.g. DNA cleavage by DNA topoisomerase II β (TopoIIβ) during transcription[130], or DNA lesion-independent mechanisms (reviewed in[131]).

All canonical histones are covalently ADP-ribosylated predominantly at the basic amino-terminal regions, although the extent of ADP-ribosylation depends on the chromatin status and the tested conditions (reviewed in[132]). In general, it is difficult to detect and localize histone ADP-ribosylation in vivo because only a small fraction of

the total histones is modified (a few percent). Thus, developing techniques that can be used to localize and characterize the sites of chromatin ADP-ribosylation in cells during different conditions is critical for further understanding the function of chromatin-associated ADP-ribosylation.

Results and Discussion

Immunoaffinity precipitation with mono- or polyclonal antibodies against poly-ADP-ribose (PAR) have been used to capture PARylated proteins for their subsequent identification[133,134], and to visualize PARylation by immunohistochemistry. However, due to the very poor affinity of these antibodies for formaldehyde-fixed extracts, PARylation of chromatin cannot be reproducibly localized with the conventional chromatin immune precipitation (ChIP) protocol or require cell-type specific optimizations of the protocols to reduce the high background. We therefore developed a new and robust method to identify chromatin-associated protein PARylation at defined loci and for whole genome analysis by combining chromatin affinity precipitation (ChAP) with qPCR, high-throughput sequencing or ChIP (Fig. 1a).

The wild-type WWE domain of the ubiquitin ligase RNF146 and the macrodomain Af1521 from archaebacteria, expressed as GST-fusion protein and bound to glutathione beads, very specifically pull down *in vitro* PARylated ARTD1 and H3 compared to their mutated counterpart (Fig. 1b). Moreover, only PARylated proteins, but not proteins MARylated by ARTD10, bound to the RNF146 WWE domain (Supplementary Fig. 1a-c). To investigate chromatin-associated ADP-ribosylation, A549 cells were either mock-treated or exposed to a sublethal dose of hydrogen peroxide (H_2O_2) (Supplementary Fig. 2a). Detection of H_2O_2-induced PAR formation by immunofluorescence, or pull down experiments using the RNF146 WWE domain and subsequent detection of PAR by immunoblotting was only possible when cells were fixed with 4% formaldehyde, a concentration that, compared to 1% formaldehyde, importantly also inhibits lysis-induced PAR-formation and prevents PAR degradation during cell lysis (Supplementary Fig. 2b-d, [135]). The isolated chromatin was then sonicated to approximately 150-250 bp fragments and subsequently enriched using either the RNF146 WWE domain or the macrodomain Af1521. Using these conditions, both domains selectively enriched the 4% formaldehyde-fixed ADP-ribosylated chromatin fraction, which contained ARTD1 (Fig. 1c and Supplementary Fig. 3a). Importantly, enrichment of the chromatin fraction was very specific, since the PAR signal was only observed when cells where treated by H_2O_2, could only be detected with the wild-type but not mutant RNF146 WWE or macrodomain Af1521 and was dependent on protein ADP-ribosylation, as the signal was completely abrogated by pretreatment of the cells with the ADP-ribosylation inhibitor ABT-888 (Fig. 1c).

To test whether the RNF146 WWE domain can be successfully used to pull down ADP-ribosylated chromatin and allowing subsequent analysis of the underlying DNA by qPCR, the RNF146 WWE-enriched chromatin fraction from mock- and H_2O_2-treated cells was analyzed for three regions with different chromatin features: the transcription start site (TSS) of the house keeping gene *GAPDH*, representing a euchromatic region with active histone marks, the promoter of the *IL1β* gene representing a compacted chromatin region with repressory histone marks and the heterochromatic alpha satellite (αSAT) regions (Fig. 1d and Supplementary Fig. 3b-d). The analysis revealed that only upon H_2O_2 treatment the wild-type, but not the ADP-ribose binding-deficient mutant WWE domain allowed the amplification of the indicated DNA regions, strongly suggesting that these chromatin loci are ADP-ribosylated. Moreover, while only very little ADP-ribosylation was observed at the TSS of the expressed *GAPDH*, a higher signal was observed for the *IL1β* promoter, and a very strong signal was obtained for the heterochromatic alpha satellite regions, suggesting that the extent of ADP-ribosylation depends on the chromatin context (Fig. 1d). Similar results were obtained using the macrodomain Af1521, although the ADP-ribosylation levels where higher at the TSS of GAPDH, which may be due to the fact that the macrodomain Af1521 detects PARylated and MARylated proteins (Supplementary Fig. 3e). The same experiments with the 4% formaldehyde fixed chromatin, but using two currently available anti PAR antibodies revealed that also under these conditions, where induced PAR formation is prevented from degradation, the ChIP signals were not specific, since also a signal for ABT-888 treated samples was detected or even higher compared to non-inhibitor treated samples (Supplementary Fig. 4a and b). Interestingly, a ChAP signal was retrieved solely at 10 min after cell stimulation, while the signal already dropped down to background after 30 min of H_2O_2 stimulation, in line with the rapid decay of H_2O_2-induced ADP-ribosylation as observed by immunofluorescence (Supplementary Fig. 5a). Moreover, the intensity of the ChAP signal was dependent on the H_2O_2 concentration used, indicating that quantitative changes in chromatin ADP-ribosylation can be detected by the novel technique (Supplementary Fig. 5b).

The observed increase in ChAP signal induced by H_2O_2 was specific for ADP-ribosylation, since co-treatment of cells with ABT-888 completely abrogated the enrichment (Fig. 1e). Furthermore, to test the contribution of ARTD1 to the detected

signal, we knocked down ARTD1 by shRNA transduction before stimulating the cells with H_2O_2. All signals detected in the 3 different regions assessed were strongly reduced to background by ARTD1 knock-down, illustrating the key role of ARTD1 in PAR induction after H_2O_2 and further demonstrating the high specificity of the method (Fig. 1e). To gain further insight into the composition of the chromatin associated with ADP-ribosylated proteins, we analyzed the chromatin association of ARTD1 by conventional ChIP or by ChAP-ChIP the material from Fig. 1d (after releasing it from the beads by SDS) with antibodies specifically immunoprecipitating ARTD1 (Fig. 1f). These experiments clearly demonstrated that ARTD1 was associated with the ADP-ribosylated chromatin, indicating that the H_2O_2-induced and ARTD1-mediated PARylation did not repel it from the chromatin under the tested conditions. Comparable analysis for H3 revealed that H3 remained also associated with the chromatin under the tested conditions (Fig. 1g) and that ADP-ribosylation is associated to regions with high ARTD1 and nucleosome content (Fig. 1f and g). To investigate the functional consequence of chromatin ADP-ribosylation, we treated cells with H_2O_2 in presence or absence of ABT-888 and tested the DNA accessibility at the same chromatin loci using CHART-PCR[136]. ADP-ribosylation substantially increased the accessibility for the MNase digestion, indicating that ADP-ribosylation reduced chromatin compaction and increased accessibility at the sites of chromatin ADP-ribosylation (Fig. 1h).

To define the genome-wide ADP-ribosylation signature induced by H_2O_2, we performed a ChAP-Seq experiment. The isolated DNA was deep sequenced after a ChAP and the retrieved reads filtered and computed in order to define peaks of ADP-ribosylated chromatin. No significant peak was identified under unstimulated conditions, indicating that either no PARylation is present in cells under basal conditions or that the amount is too low to be detectable (Fig. 2a upper panel). However, upon H_2O_2 treatment, the high-throughput sequencing analysis identified 6'633 unique peaks with an average size of 500 bp (Fig. 2d lower panel, b and c). ChAP-qPCR analysis using the macrodomain AF1521 covering up to 10 kb up- or downstream of an assigned peak confirmed that the detected chromatin ADP-ribosylation peaks indeed covered only a distinct chromatin region (Fig. 2d).

While the vast majority of the peaks were located in intergenic regions and introns, only few peaks fell into TSSs, promoters or exons, indicating that H_2O_2-induced ADP-ribosylation occurs less in these regions close to genes (Fig. 2e). When

analyzing the underlying sequences, chromatin ADP-riboslyation was mainly found to be associated with SINE, LINE or αSAT repetitive elements ([137], Fig. 2f). Enrichment of the latter mirrored our ChAP-qPCR data, where the highest ChAP signals were also obtained for αSAT (Fig. 1d; Supplementary Fig. 3e).

To further explore the applicability of the new ChAP technique for a different cell type and addressing another biologically important questions, we characterized chromatin ADP-ribosylation in cells undergoing adipogenesis. We have recently reported that the inhibition of ADP-ribosylation or depletion of ARTD1 severely hampers PPARγ-dependent gene expression in differentiated 3T3L1 cells [138]. When we applied the ChAP method to differentiated adipocytes, we were able to specifically enrich ADP-ribosylated chromatin fragments containing nucleosomes (i.e. H3), ARTD1 and protein-associated PAR (Fig. 3a). Further analysis of the DNA associated with the ADP-ribosylated chromatin revealed a high PAR signal on the PPARγ-response elements of PPARγ target genes such as *aP2* and *adiponectin* and no such signal for *CD36* or *K19,* a control gene not dependent on PPARγ (Fig. 3b), indicating that not all promoters of PPARγ target genes are ADP-ribosylated to the same extent or at the same time. Over night pretreatment of adipocytes with ABT-888 prevented any enrichment, confirming again that the enriched chromatin was ADP-ribosylated and that the ChAP method was also specific using this cellular system (Fig. 3b). ChIP analysis of ARTD1 and PPARγ on *aP2* revealed that ARTD1 and PPARγ are both recruited to the *aP2* promoters during differentiation (Fig. 3c, d0 versus d7). ChAP-ChIP analysis of the same regions revealed that while ARTD1 levels were unchanged, a strong increase of PPARγ binding to the ADP-ribosylated *aP2* promoter was observed, indicating that chromatin ADP-ribosylation helps recruiting PPARγ to this promoter site (Fig. 3d). This was not the case for *CD36*, a gene less associated with PARylation **(**Fig. 3b-d).

In summary, we developed a novel highly specific method allowing genome-wide ADP-ribosylated chromatin regions to be identified in different cellular paradigms of ADP-ribosylation induction. The specificity of the developed PAR-ChAP method was shown i) by detecting a PAR-specific ChAP signal only, when PAR formation was induced in cells, ii) by enrichment only with the wild-type RNF146 WWE domain or the macrodomain Af1521, but not with mutants harboring single mutants abrogating binding to ADP-ribosylation and iii) by loosing the signal

upon treatment of the cells with the ADP-ribosylation inhibitor ABT-888 or knocking down the ARTD1, the enzyme responsible for PAR formation under the tested conditions. We expect that the identification of ADP-ribosylated chromatin loci using this new methodology will significantly promote the discovery of new insights into how ARTD1 regulates chromatin plasticity during different biologically important processes.

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Authors contributions: G.B. planned, performed and evaluated the experiments, prepared figures and revised the manuscript. M.O.H. designed the experiments, supervised the project and wrote the manuscript.

Methods

In vitro ADP-ribosylation: Auto-modifications assays were performed as previously described[138]. Briefly: ARTD1, ARTD2 or ARTD10 were incubated with the reported concentration of [^{32}P]NAD$^+$ (PerkinElmer) or 100 μM cold NAD$^+$ for 1 h at 30°C; ADP-ribosylated proteins were incubated together with WWE domain (see ChAP chapter); resolved on SDS-page and either exposed on X-ray films ([^{32}P]NAD$^+$) or immunoblitted with the reported antibodies. Trans ADP-ribosylation of histones was carried out adding to the ARTD1 reaction 1 μg of histones mix (Roche 10223565001).

GST-bait expression: BL21 competent E. coli cells were transformed with a GST-WWE or GST-Af1521 coding plasmid and grew in LB medium and resistance till OD reached 0.5. Cells were induced with 100 μM IPTG and incubated for additional 3h at 30°C. Bacteria were then lysed in M buffer 750 (50 mM Tris-HCl pH 8, 750 mM NaCl, 0.1% NP-40, 5 mM EDTA, proteinase inhibitor (Roche)) through French press; lysates were cleared by ultra centrifugation and GST-baits were quantified on SDS-page using BSA as standards. 600 μl slurry glutathione magnetic beads (Pierce) were incubated with 2 mg GST-bait in M buffer 750 for 3 h; washed then 3 times in M buffer 750 (5 min each); 1x wash-2 (see ChIP chapter); eventually resuspended in 1 ml of M buffer 500 (50 mM Tris-HCl pH 8, 500 mM NaCl, 0.1% NP-40, 5 mM EDTA) and stored at 4°C.

Cells culture and treatment: A549 cells and 3T3L1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (PAA, supplemented with 1% (v/v) Penicillin/Streptavidin and 10% (v/v) fetal calf serum (Gibco). shMock and shARTD1 cells were generated as previously described [90] and maintained in medium with 1.5 μg/μl puromycin (Puromycin). A549 cells were treated in PBS with 1mM (when not otherwise stated) H_2O_2 (Sigma) and incubated for 10 min. before proceeding with downstream protocols. Cells inhibited with PARP-inhibitor were preincubated with 1μM ABT-888 (ENZO) for 30 min. and H_2O_2 stimulation was carried out in the presence of fresh inhibitor. 3T3L1 cells were differentiated as previously described[138] and processed at d7. 3T3L1 cells inhibited with PARP-inhibitor were preincubated with 1μM ABT-888 12 h before collection.

Immunofluorescence:. $1x10^5$ A549 cells were seeded on coverslips; 24 h later treated in H_2O_2 and processed as previously described[139].

Antibodies: anti-PAR 10H (home made); anti-PAR (ALX-210-890) (ENZO); anti-PARP (sc-7150) for western blot, anti-PPARγ rb (2443S), GST (Z-5), normal rabbit IgG (Santa Cruz); anti-PARP (46D11) for ChIP (Cell Signaling); histone H3 (Abcam); acetyl-Histone H4, trimethyl-HistoneH3 (Lys27), trimethyl-HistoneH3 (Lys9) (07-442) (Millipore); acety-Histone H3 (Upstate).

Chromatin immuno precipitation (ChIP):. 1x15cm dish containing $4x10^6$ A549 cells (over night seeding) or confluent 3T3L1 per each condition were used. Cells were crosslinked in cold PBS containing 4% formaldehyde (Sigma) for 10 min. at 4°C; washed in cold PBS; and collected in 1ml of cold lysis buffer (50 mM Tris HCl pH 8, 400 mM NaCl, 1% Triton-X, 5 mM EDTA, 1% SDS). A549 cells were lysed one time whereas for 3T3L1 it was necessary to repeat the lysis step 5 times with 3 min. 5k g centrifugation in between in order to get rid of the cytoplasmic lipids. Cell pellet was then resuspended in D buffer (50 mM Tris HCl pH 8, 1% SDS, 5mM EDTA) and chromatin fragmented to 150-250 bp through sonication (Bioruptor). Chromatin was then cleared of debris by 10 min. centrifugation at 7k rpm at room temperature; and quantified as follow: 2 µl cleared chromatin were diluted in 18 µl TE buffer containing 20 U of proteinase K; reactions were incubated at 37°C for 1h; DNA was quantified with NanoDrop (Thermo Scientific) and run on agarose gel to check shearing efficiency. 3 µg of chromatin were saved as input and 30 µg per pull-down were diluted in ChIP buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA, 0.01% SDS, 1.1% Triton-X, proteinase inhibitor (Roche)); incubated with 2 µg of the reported antibody over night; and the following morning 10 µl of Protein A Dynabeads (life Technologies) were added and incubated for additional 4h. Following binding, beads were washed 2x wash-1 (0.1% SDS, 1% Triton X, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH8), 1x wash-1high salt (0.1% SDS, 1% Triton X, 2 mM EDTA, 500 mM NaCl, 10 mM Tris-HCl pH 8), 2x wash-2 (0.25 M LiCl2, 0.5% NP-40, 1 mM EDTA, 0.5% Na-deoxycholate) and 2x TE (10 mM Tris-HCl pH 8, 5 mM EDTA). Protein-DNA complexes were digested on beads in 250 µl of buffer D supplemented with 400 mM (NaCl) and 20 U proteinase K for 1 h shaking at 42°C

and decrosslinked over night at 65°C. DNA was then extracted with phenol-chloroform; precipitated in ethanol; resuspended in water and analyzed by real-time PCR using SYBR Green and the Rotor-Gene 3000 (Qiagen).

Chromatin affinity precipitation (ChAP): The following description refers to a single pull down, cells and reagents were scaled up accordingly in case more reactions were carried out simultaneously. 1x15 cm dish containing $4x10^6$ A549 cells (over night seeding) or confluent 3T3L1 were processed as for ChIP: cells were crosslinked in 4% formaldehyde; lysed in lysis buffer; chromatin was resuspended in 200µl of buffer D; sonicated; and cleared of debris (all these steps as in ChIP chapter). Chromatin was then diluted in 10 ml of cold M buffer 500 (50 mM Tris-HCl pH 8, 500 mM NaCl, 0.1% NP-40, 5 mM EDTA, proteinase inhibitor (Roche)) and incubated for 3h together with 100µl beads-bound GST-WWE or GST-Af1521. After binding, beads were washed 1x in M buffer 500 followed by washes as reported in the ChIP chapter.

ChAP-WB: Proteins were released from beads and protein complexes decrosslinked by boiling in Laemmli-buffer for 30 min.

ChAP-qPCR: Protein-DNA complexes were eluted and processed as described in the ChIP chapter.

ChAP-Seq: 1 ng of purified DNA (or the corresponding volume for the negative control samples) was used to generate barcoded library using Ovation Ultralow Library Prep kit (NuGen) according to manufacture protocol. Hybridized DNA was sequenced on Illumina HiSeq 2000 platform to generate paired-end 100 bp reads. Raw reads were aligned to the human genome (hg19 assembly) using Bowtie2, allowing one mismatch in the seed region of 31 nt and selecting the best alignment based on alignment scores in case of multiple hits. PCR duplicates were identified and discarded using Picard. The resulting read alignments were used to identify genomic regions with significant read enrichments (peaks) using the HOMER software in both factor and histone mode to statistically test for enrichments in narrow and broad genomic regions, respectively. The peak calling procedure was carried out by comparing the wild-type VS mutant samples, considered as background. To quantify

read enrichment within different classes of repetitive elements the genomic coordinates of different classes of repetitive elements spanned by the UCSC genome browser RepeatMasker were retrieved and used to construct a Bowtie index of repetitive sequences. The reads of each sample were aligned to this new reference. A read was only counted once per class regardless if it aligned to multiple sequences within the same class. GO analysis was performed using DAVID software[140].

ChAP-ChIP: Proteins were released from beads by 2x 15 min. incubation in 100 µl of buffer D shaking at 37°C. Elution was then diluted in ChIP buffer and ChIP performed as previously described.

CHART-PCR: Cells were fixed as for ChIP, washed and collected in cold PBS, and counted. $1x10^6$ cells were gently resuspended in 1 ml of MNase lysis buffer (10 mM Tris-HCl pH8, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 0.15 mM spermine, 0.5 mM spermidine) and incubated on ice for 5 min. followed by 10 min. 2 g centrifugation at 4°C. Cells were resuspended in 1 ml of MNase digestion buffer (10 mM Tris-HCl pH 8, 15 mM NaCl, 0.15 mM $MgCl_2$, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2.5 mM $CaCl_2$) and per each digestion reaction 100 µl were used. Digestion was carried out at room temperature with 3 unit of MNase (Roche) for 0, 1, 5, 10 and 20 min. and reaction was stopped adding 100 µl of MNase STOP solution (10 mM EDTA, 2 mM EGTA, 2% SDS) and 20 U proteinase K. Protein digestion, decrosslinking and DNA isolation were performed as described in the ChIP chapter. DNA was analyzed by qPCR and its accessibility was assessed by comparing it to undigested DNA.

Primers.

Locus	Forward	Reverse
GAPDH	CGTAGCTCAGGCCTCAAGAC	GCTGCGGGCTCAATTTATAG
IL1beta	GTCTTCCACTTTGTCCCACA	TGACAATCGTTGTGCAGTTG
AlphaSATELLITE	CTG CAC TAC CTG AAG AGG AC	GAT GGT TCA ACA CTC TTA CA
Chr17 -10 kb	GAGCACAGCGCCACCA	GCACAGCCCCTGCACA
Chr17 -5 kb	CCTCACGTGGCTGCCC	CCTTCCTGCCAGGCTGC
Chr17 -1 kb	AGCCACCAGCCAACGG	GAGGGCAAAGCGCCCA
Chr17 peak kb	GGGAAACTCTGCGCCACTAT	CAGTGTCTCATGCTGGTGT
Chr17 +1 kb	TCCGATAGGCCGGTTTTGAC	GGACGGAGTCTCTTCTCCCT
Chr17 +5 kb	GGAGGAACAGACAGGATGGC	TTCCAGGGCAGGGACTATGA
Chr17 +10 kb	CTTCCACCTGGGAACCTGAG	CCAGGAAGCAGGCTGATTGA

Fig. 1: The RFN146 WWE specifically pulls-down *in vivo* ADP-ribosylated proteins cross-linked to DNA.

(**a**) Schematic overview of the ChAP workflow. (**b**) Western blotting analysis of ARTD1 and histones (i.e. H3) ADP-ribosylated *in vitro* and pulled down with wild-type or mutant WWE and Af1521. The original image was edited by removing 1 lane. (**c**) Western blotting analysis of PAR and ARTD1 using chromatin prepared for ChAP as described in M&M from A549 cells untreated or treated with H_2O_2 and/or ABT-888, inhibitor of ADP-ribosylation, and enriched by ChAP with WWE. (**d**) qPCR analysis of *GAPDH* TSS, *IL1β* promoter and *αSAT* of material enriched as in (**c**). (**e**) qPCR analysis of shMock and shARTD1 cells stimulated with H_2O_2 and/or ABT-888 and analyzed as in (**d**). (**f,g**) qPCR analysis of formaldehyde-fixed extracts from cells treated with H_2O_2 and enriched by ARTD1 (**f**) or histone H3 (**g**) ChIP or ChAP-ChIP. (**h**) CHART-PCR of cells treated with H_2O_2 and/or ABT-888.

Fig. 2: ADP-ribosylated proteins peak at DNA repetitive sequences.

(**a**) Genome browser screen shot of a representative ADP-ribose peak (ID 12536) over a repetitive sequence. (**b**) Number of unique reads sequenced per each sample. (**c**) Average peak length of H_2O_2 treated sample. (**d**) qPCR analysis of the region surrounding peak ID 12536 using formaldehyde-fixed extracts from A549 cells treated with H_2O_2 and/or ABT-888 and enriched by ChAP with Af1521. (**e**) Distribution of H_2O_2 peaks relative to the closest gene. (**f**) Percentage of H_2O_2 peaks overlapping with repetitive sequences.

Fig. 3: ADP-ribosylated proteins localize at the PRRE of a subset of PPARγ target genes.

(**a**) Western blotting analysis of PAR, ARTD1 and histone H3 using formaldehyde-fixed extracts from undifferentiated (d0) or differentiated (d7) enriched by ChAP with WWE. (**b**) qPCR analysis on the PRRE of PPARγ target genes *AP2*, *Adiponectin* and *CD36* and on *K19* promoter of material enriched as in (**a**), 12h ABT-888 inhibited cells were included in the analysis. (**c**) ARTD1 and PPARγ occupancy on *AP2* and *CD36* PRRE at d0 and d7. (**d**) qPCR analysis of ChIP and ChAP-ChIP of ARTD1 and PPARγ on *AP2* and *CD36* PRRE at d7.

Supplementary Fig. 1: the RNF146 WWE domain recognizes poly- but not mono-ADP-ribosylated proteins.

(**a,b**) Autoradiography and coomassie staining of ARTD1 (**a**) or ARTD10 (**b**) *in vitro* modified in the presence of 100 nM $^{32P}NAD^{+}$ and incubated with wild-type (wt) GST-WWE, or with the iso-ADP-r binding deficient mutant R163A or the binding proficient mutant R161A; protein complexes were then resolved on SDS page and exposed on X-ray film. (**c**) Autoradiography and coomassie staining of ARTD1, ARTD2 and ARTD10 auto-ADP-ribosylated *in vitro* in the presence of 100 μM $^{32P}NAD^{+}$ and enriched with GST-WWE as in (**a**).

Supplementary Fig. 2: 1 mM H_2O_2 treatment for 10' does not impair cell viability or growth and fixation with 4% formaldehyde prevents PAR degradation and artifact generation.

(**a**) Cell count of 24h or 48h post 10' H_2O_2 treatment followed by recovery. Cells were treated when sub-confluent (left) of confluent (right). (**b**) PAR immunofluorescence (red) of cells left untreated or H_2O_2-treated and fixed in methanol:acetic acid (MeOH), 1% or 4% formaldehyde (Form.). DAPI (blue) was used as nuclear staining. (**c**) ARTD1 western blotting analysis of untreated and not-fixed A549 cells lysed in lysis buffer (left) or lysis buffer containing ADP-ribosylation inhibitor (PJ34, right). Protein extracts were enriched with WWE-domain. (**d**) PAR and ARTD1 western blotting analysis of cells left untreated or treated with H_2O_2 and/or ABT-888 and processed for ChAP.

Supplementary Fig. 3: The Af1521 macrodomain specifically enriches *in vivo* ADP-ribosylated cross-linked chromatin.

(**a**) Western blotting analysis of PAR and ARTD1 using chromatin prepared for ChAP as described in M&M from A549 cells treated with H_2O_2 and enriched by ChAP with WWE or Af1521. (**b-d**) ChIP analysis of histone H3 (**a**), ARTD1 (**b**), 3 histone H3 modifications (**c**) at *GAPDH* TSS, *IL1β* promoter and on *αSAT* regions.

(**e**) ChAP analysis of *GAPDH* TSS, *IL1β* promoter and *αSAT* of material enriched as in (**a**).

Supplementary Fig. 4: Anti-PAR antibodies are not suitable for ChIP under the tested conditions.

(**a,b**) qPCR analysis using chromatin prepared for ChAP as described in M&M from A549 cells treated with H_2O_2 and/or ABT-888 and enriched by ChIP with 10H (**a**) or a polyclonal anti-PAR antibody (**b**).

Supplementary Fig. 5: H_2O_2-induced chromatin ADP-ribosylation is reversible and PAR-ChAP signal intensity depends on the H_2O_2 concentration

(a) qPCR analysis using chromatin prepared for ChAP as described in M&M from A549 cells left untreated (-), treated for 10' with H_2O_2, treated for 10' with H_2O_2 and let recover for additional 20' (30') or for additional 80' (90') and then enriched by ChAP with WWE. **(b)** qPCR analysis using chromatin prepared for ChAP as described in M&M from A549 cells left untreated (-) or treated for 10' with 0.2 mM or 1 mM H_2O_2.

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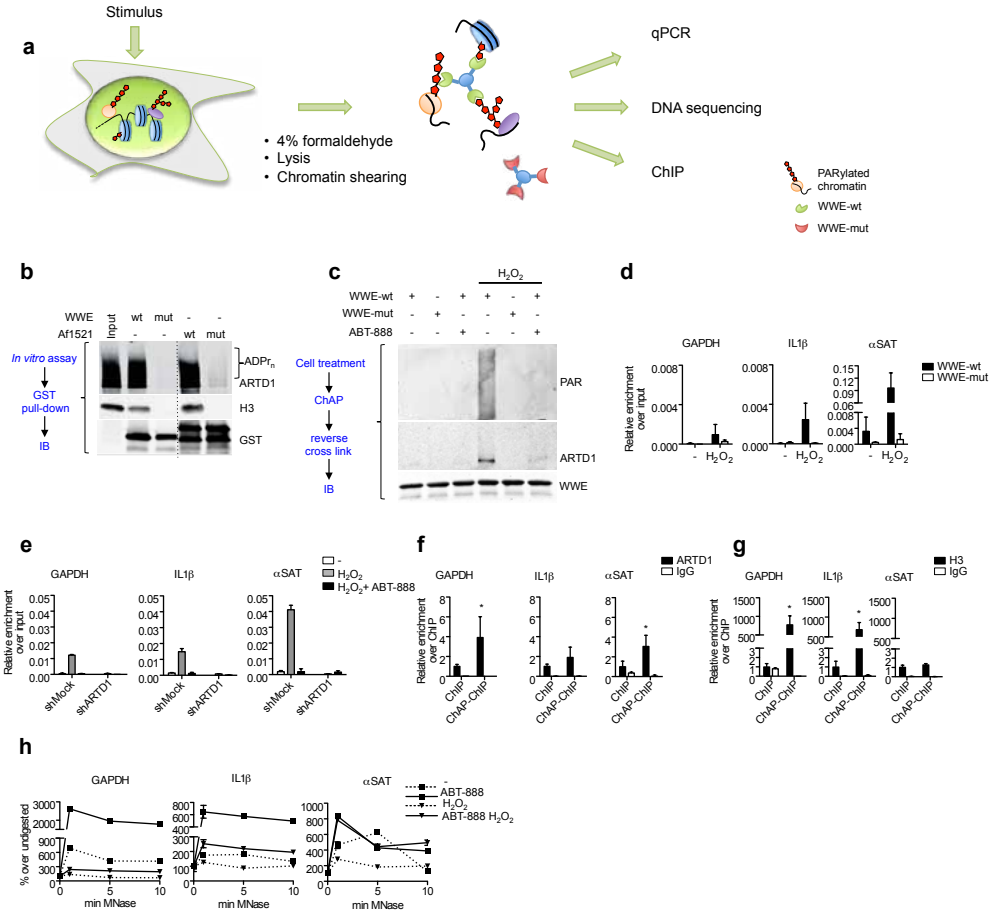
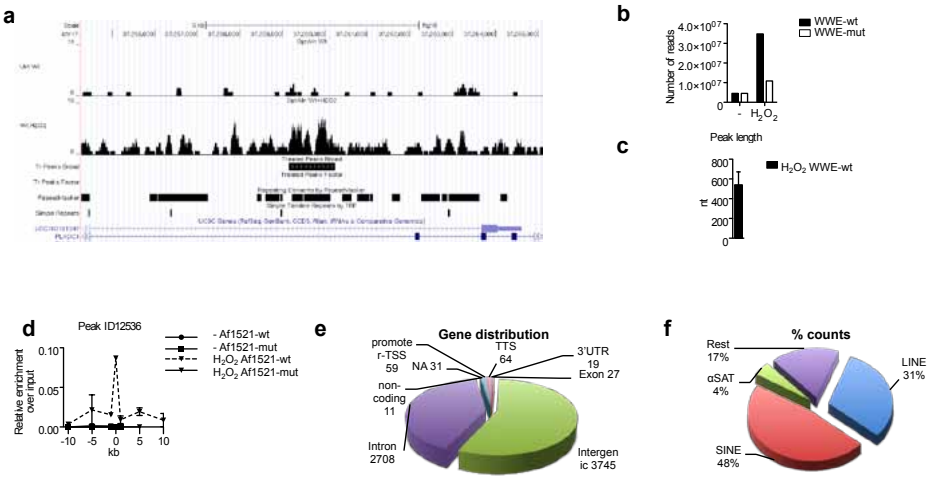
a

Bartolomei Figure 1

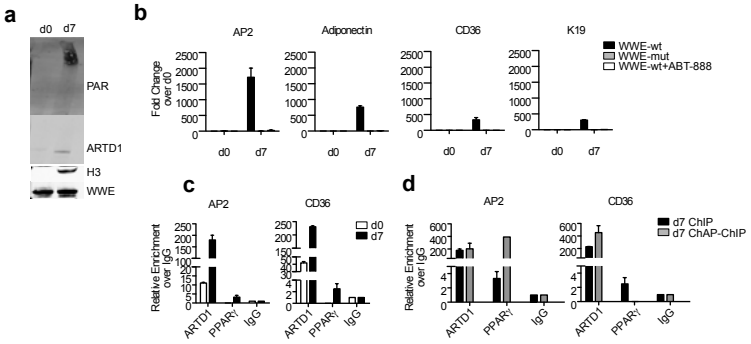


Bartolomei Figure 2

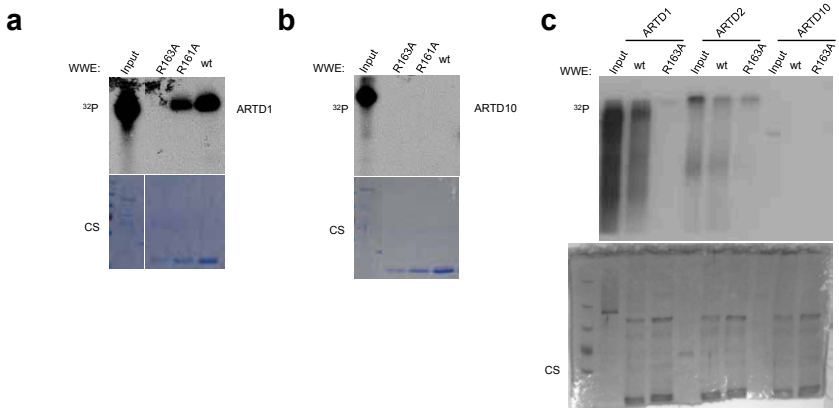


Bartolomei

Figure 3



Bartolomei Suppl. Figure 1



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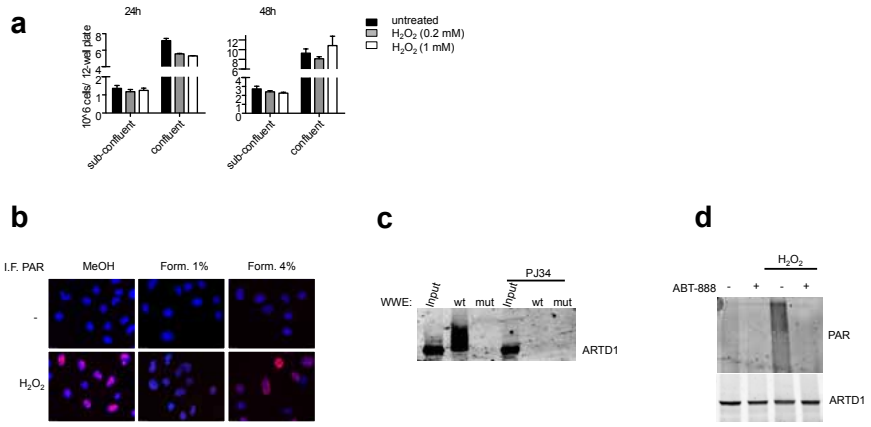
Bartolomei Suppl. Figure 2



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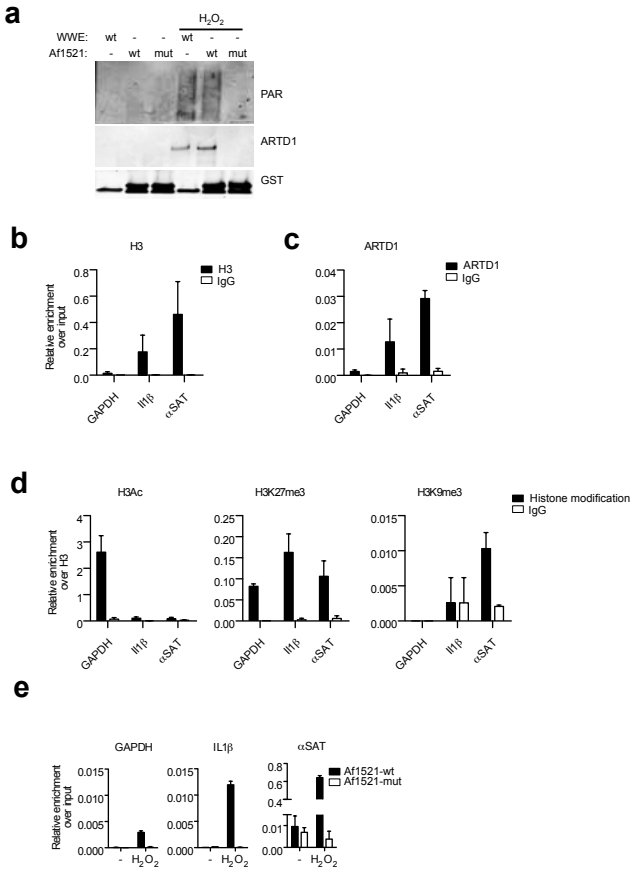

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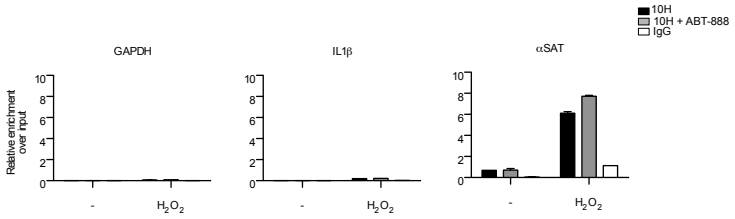
Bartolomei Suppl. Figure 4

a



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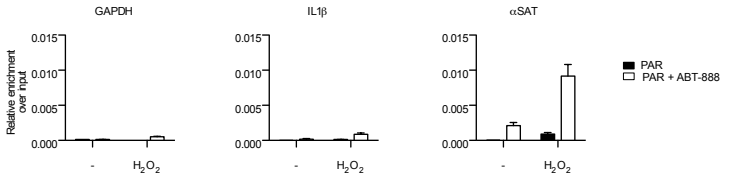

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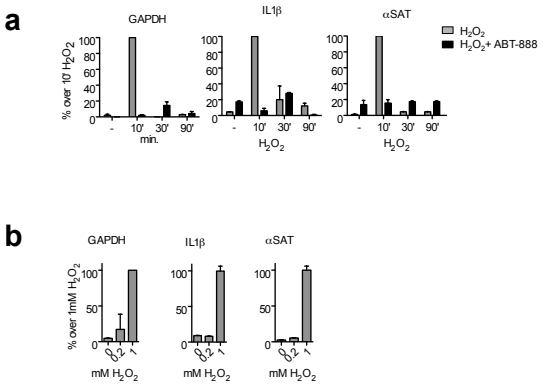
Bartolomei Suppl. Figure 5



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3.3 Unpublished results

3.3.1 H_2O_2-induced ADP-ribosylation does not change chromatin composition and is associated with constitutive heterochromatin

Since heavily auto-ADP-ribosylated ARTD1 or trans-modified nucleosomes have been shown to be evicted from chromatin, we sought to assess whether H_2O_2-induced ADP-ribosylation provoked ARTD1 or nucleosome eviction from the chromatin[25,141,142]. We therefore induced nuclear ADP-ribosylation by treating A549 cells with H_2O_2 or prevented it by treating cells with ABT-888 and subsequently performed ChIP analysis for ARTD1 and nucleosome (i.e. histone H3) (Fig.4A,B).

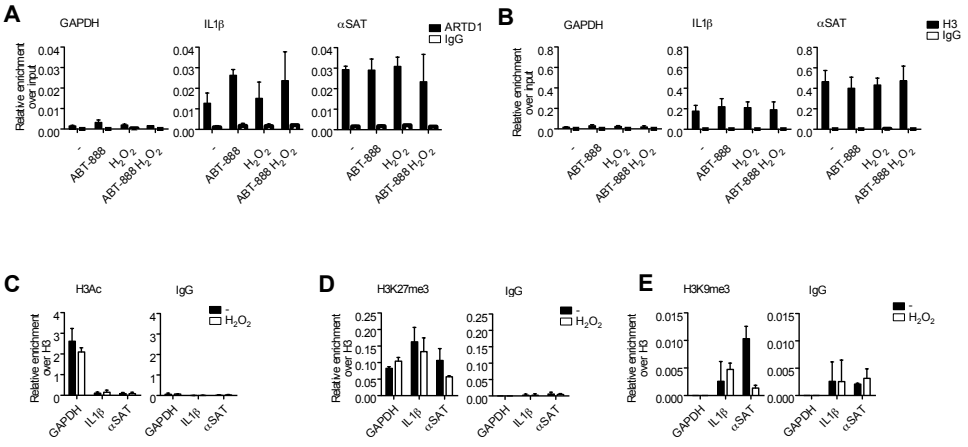

Figure 4: H_2O_2-induced ADP-ribosylation does not change the chromatin composition.

(A) ARTD1 ChIP analysis of A549 upon stimulation with 1mM H_2O_2 for 10min. ADP-ribosylation inhibited samples were pre-incubated for 30min with 1μM ABT-888. Values of *GAPDH* promoter, *IL1β* promoter and *α-SAT* regions are shown. (B) Histone H3 ChIP analysis as in (A). (C-E) ChIP analysis on histone modification of untreated cells or after H_2O_2 treatment.

ChIP experiments showed that, in the same tested regions as in the submitted manuscript, the density of ARTD1 and the nucleosome correlated. The higher the histone H3 signal (i.e. chromatin compaction), the more abundant was ARTD1 (Fig.4A,B). In addition, although ADP-ribosylation was described to induce protein eviction from the chromatin, we did not observe any change in ARTD1 or histone H3 occupancy independently whether cells were treated only with H_2O_2 or ABT-888 or in combination (Fig.4A,B). We then expanded the ChIP analysis for 3 histone modifications, including histone H3 acetylation (H3Ac), an open chromatin marker,

H3K27me3, a facultative heterochromatin marker, and histone H3 lysine 9 tri-methylation (H3K9me3), a constitutive heterochromatin marker (Fig.4C-E). The ChIP data revealed that the *GAPDH* promoter was enriched for H3Ac while the *IL1β* promoter and *α-SAT* regions were both enriched for H3K9me3 (Fig.4C,E). In addition, H_2O_2 treatment did not alter the histone marks at the *GAPDH* or *IL1β* promoter indicating that H_2O_2-induced ADP-ribosylation does not change the nucleosome modification at these 2 promoters. In contrast, we observed a drop-down of H3K27me3 and H3K9me3 modifications after H_2O_2 treatment at the *α-SAT* regions, suggesting that the *α-SAT* regions may be more prone to regulation by ADP-ribosylation upon H_2O_2 treatment than the other tested regions. Additional experiments are needed to mechanistically explain the effect of H_2O_2-induced ADP-ribosylation at the *α-SAT* regions.

To gain further insight into the histone modifications associated with ADP-ribosylated chromatin, we treated A549 with H_2O_2 and enriched PARylated chromatin fragments by ChAP. We then released these fragments with SDS and performed ChIP (ChAP-ChIP) analysis for H3Ac, H3K27me3 and H3K9me3 (Fig.5A-C).

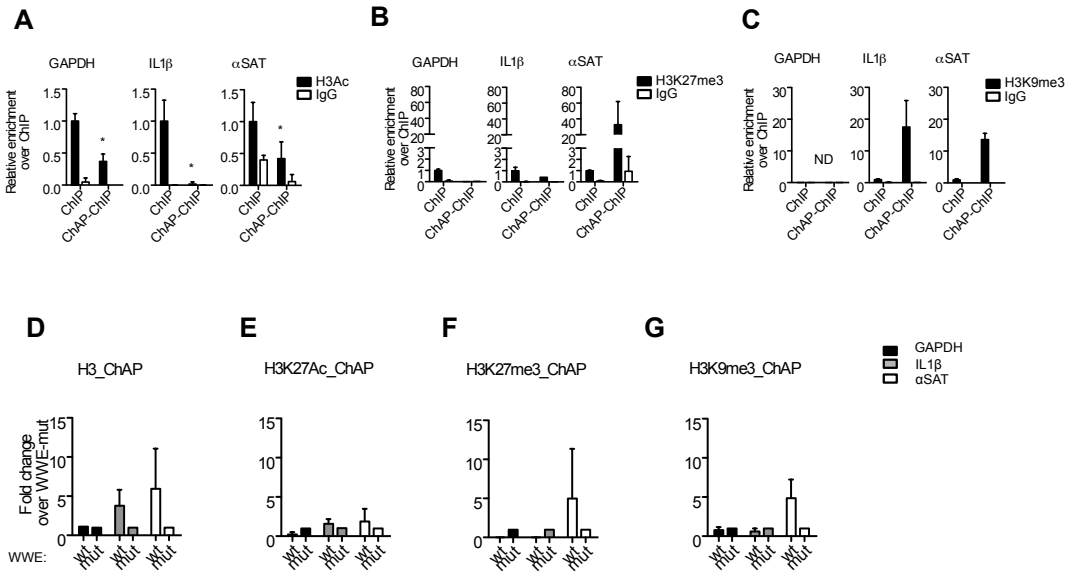

Figure 5: H_2O_2-induced poly-ADP-ribosylation preferentially associates with constitutive heterochromatin.

(A) ChIP and ChAP-ChIP comparison of histone H3Ac after cell stimulation with H_2O_2. Values of *GAPDH* promoter, *IL1β* promoter and *α-SAT* regions are shown. Values are expressed as fold change over the ChIP signal arbitrarily set as 1. (B) ChIP and ChAP-ChIP comparison of histone H3K27me3 as in (A). (C) ChIP and ChAP-ChIP comparison of histone H3K9me3 as in (A). (D-E) ChIP-ChAP analysis of the reported histone modifications at the *GAPDH*, *IL1β* promoter and *α-SAT* regions. Values are expressed as fold change over the signal of the mutant WWE domain arbitrarily set as 1.

Our analysis showed that the ChAP-ChIP signals for histone H3Ac were lower than the ChIP signals for the tested regions, indicating that H_2O_2-induced PARylation and H3Ac are mutually exclusive (Fig.5A). Similar results were obtained when we analyzed H3K27me3, although we observed a positive correlation between PARylation and H3K27me3 at the *α-SAT* (Fig.5B). These data confirm that H_2O_2-induced PARylation is differently regulated at the *α-SAT* regions compared to the *GAPDH* or *IL1β* promoter. In addition, we observed that H_2O_2-induced PARylation at the *IL1β* promoter and at the *α-SAT* regions was found with constitutive heterochromatic histone mark H3K9me3 (Fig.5C).

To confirm these ChAP-ChIP data, we reversed the experiment and performed ChIP for histone H3, H3K27Ac, H3K27me3 or H3K9me3. We then released the fragments by SDS and performed a ChAP (ChIP-ChAP) analysis using either the wild-type (wt) or mutant (mut) WWE domain (Fig.5D-G). Our analysis revealed that histone H3 was associated with ADP-ribosylation at the *IL1β* promoter and at the *α-SAT* regions but not at the open *GAPDH* promoter, confirming that H_2O_2-induced chromatin

PARylation is associated with regions with high nucleosome content (Fig.5D). Conversely, no PAR signal was associated with H3K27Ac at any of the tested regions, in line with the notion that upon H_2O_2 treatment PARylation and histone H3 acetylation are mutually exclusive (Fig.5E and A). We also observed that H_2O_2-induced PARylation was associated with H3K27me3 and H3K9me3 at the *α-SAT* regions but not at *GAPDH* or *IL1β* promoter (Fig.5F and G), partially confirming the ChAP-ChIP results (Fig.5B,C). Overall our data indicate that upon H_2O_2 treatment PARylation is preferentially associated with constitutive heterochromatic regions and negatively correlates with histone H3 acetylation.

3.3.2 H_2O_2 induced ADP-ribosylation or inhibition of ADP-ribosylation do not influence NF-κB-dependent gene expression

Inhibitors of ADP-ribosylation have been shown to protect from eccessive inflammation and cell necrosis in response to cytokine overproduction[121]. In addition, ARTD1 has been linked to NF-κB target gene transcription, but it is still under debate whether ARTD1 enzymatic acticity is required or not[64,143]. Thus, we aimed at studying the effect of ADP-ribosylation on the transcription of NF-κB target genes. A549 epithelial cells were either treated with LPS alone, a compound known to activate NF-κB, in presence or absence of the ADP-ribosylation inhibitor ABT-888. Gene expression analysis revealed that 1h LPS treatment neither induced inducible nitric oxide synthase (*iNOS*), interferon gamma-induced protein 10 (*IP10*) nor interleukin 6 (*IL6*), 3 genes regulated by NF-κB, indicating that A549 cells are not sensitive to LPS treatment (Fig.6A).

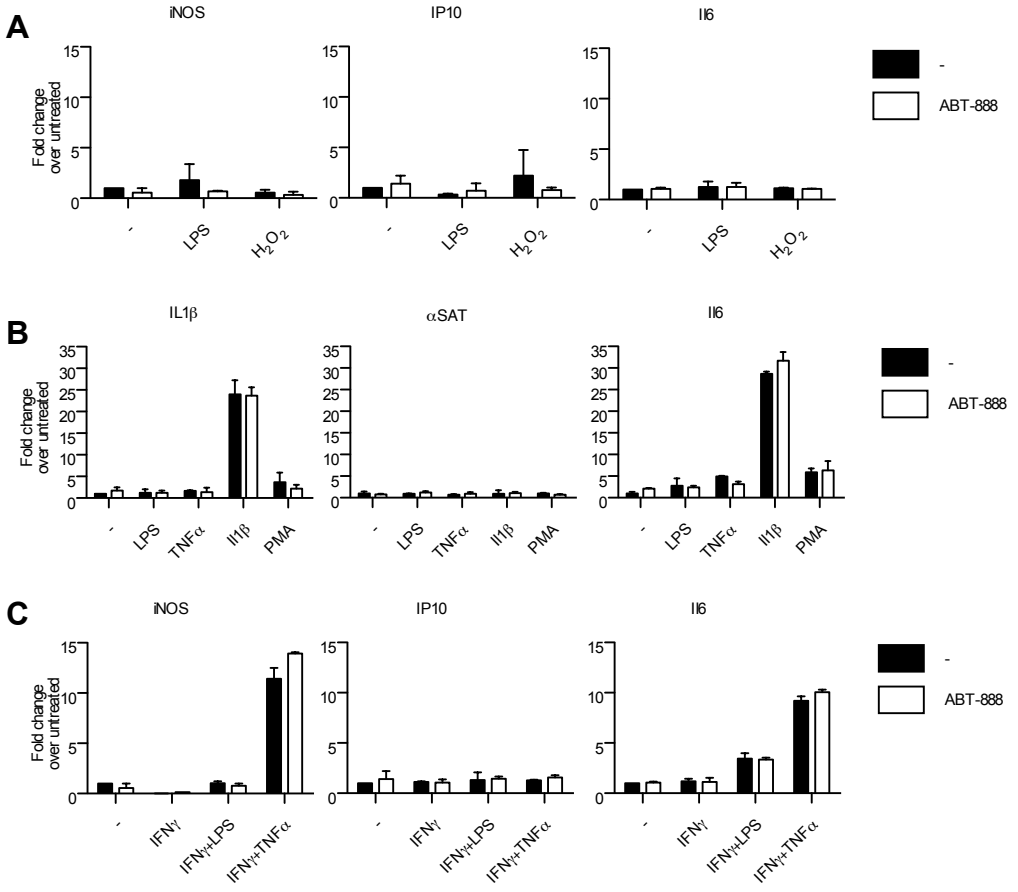

Figure 6: H_2O_2-induced ADP-ribosylation or inhibition of ADP-ribosylation do not influence NF-κB-dependent gene expression in A549 cells.

(A) Gene expression analysis of A549 upon stimulation with 1µg/ml LPS for 1h or 1mM H_2O_2 for 10min followed by 20min recovery. ADP-ribosylation inhibited samples were pre-incubated for 30min with 1µM ABT-888. (B) Gene expression analysis of cells stimulated for 4h with 10ng/ml LPS or 20ng/ml TNFα or 20ng/ml IL1β or 5ng/ml PMA. ADP-ribosylation inhibited samples were pre-incubated with 10µM ABT-888 30min before stimulation. (C) Gene expression analysis of A549 upon overnight stimulation with 10gn/ml IFNγ followed by 2h 0.1µg/ml LPS or 40ng/ml TNFα. ADP-ribosylation inhibited samples were pre-incubated with 1µM ABT-888 30min before LPS or TNFα stimulation.

Repeating the experiment by replacing LPS with H_2O_2 revealed that also H_2O_2 does not induce NF-κB target gene transcription in these cells, although the same treatment induced PAR formation (Fig.6A).

We then extended the analysis including other NF-κB-inducing stimuli such as LPS, tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL1β) or phorbol 12-myristate 13-acetate (PMA) and prolonged the treatment for 4h (Fig.6B). Cell treatment with LPS, TNFα or PMA did not induce *IL1β, IL6* or any detectable transcript across the α-satellite (*α-SAT*) regions, whereas IL1β promoted the transcription of *IL1β* and *IL6* but not of the *α-SAT*. This data show that only IL1β treatment induced NF-κB in

A549 cells. Pretreatment of cells with ABT-888 did not alter IL1β-induced gene transcription suggesting that PARylation has no direct effect on IL1β-mediated NF-κB-dependent gene expression (Fig.6B).

To further test whether ADP-ribosylation could influence the transcription of pro-inflammatory genes in A549 cells, we primed the cells with interferon gamma (IFNγ) overnight, followed by NF-κB activation via LPS or TNFα treatment for 2h (Fig.6C). IFNγ alone and in combination with LPS did not induce *iNOS*, *IP10* or *IL6* expression confirming that A549 are insensitive to LPS even when pretreated with IFNγ. In contrast, IFNγ combined with TNFα induced *iNOS* and *IL6* but not *IP-10* indicating that not all NF-κB target genes are transcribed upon stimulation with IFNγ and TNFα. Nevertheless, also under these conditions, inhibition of ADP-ribosylation did not alter gene transcription (Fig.6C).

In summary, our data indicate that ADP-ribosylation does not influence the transcription of NF-κB target genes under the tested conditions in A549 cells.

4 Discussion and Perspectives

4.1 ARTD1-mediated PARylation drives iPS cells reprogramming by enhancing SOX2 recruitment to its target gene *Fgf4*

In the published work we reported that ARTD1 and PARylation regulate fibroblasts to iPSC reprogramming at an early stage. In addition, we have mechanistically shown that ARTD1 PARylates SOX2 to enhance its recruitment to SOX2-binding sites on target genes and thus drive gene transcription. Among the regulated SOX2 target genes, we identified *Fgf4* as key regulator of iPSC reprogramming. Indeed, early administration of exogenous FGF4 protein into the medium of ARTD1-/- or ADP-ribosylation inhibited fibroblasts restored the reprograming efficiency to the levels observed for wild-type cells. In summary, the collaboration with the laboratory of Dr. Cinelli has mechanistically linked ARTD1 and PARylation to an efficient reprogramming of fibroblast to iPS cells. This part of my thesis thus extends our knowledge of iPSC reprogramming, a process of great relevance in regenerative medicine.

PAR has been described to regulate the recruitment of transcription factors to their regulatory element in actively transcribed genes. For example, it has been shown that PAR functions on PPARγ target genes as a cage to enhance PPARγ ligand binding and cofactor exchange during adipogenesis[138]. Similarly, ARTD1 has been reported to promote the NFAT-mediated transcription of *interleukin 2* by ADP-ribosylating NFAT DNA-binding domain and increasing its avidity for the DNA, [144]. Thus it seems that PAR supports gene transcription through different mechanisms that are dependent on the context and on the signaling that induced PAR formation. For example, ADP-ribosylation is generated both in response to stress stimuli and as a consequence of more physiological processes such as adipogenesis. The different natures of the initial stimulus must be then mirrored on the chromatin-associated PAR, and on its mechanism of action.

Upon transduction of somatic cells with the Yamanaka factors, cells undergo extensive chromatic and epigenetic changes to reset their cell-identity and to acquire stemness[145,146]. In this process, SOX2 is a key transcription factor that cooperates with the octamer-binding transcription factor 4 (OCT4) to induce the expression of

pluripotent genes, leading to the formation of pluripotent stem cells[147]. However, somatic cell-reprograming with the Yamanaka factors is currently a very inefficient process and the mechanisms that promote and sustain the expression of pluripotent genes are yet poorly understood[147,148]. Although no previous study has reported a functional contribution of ARTD1 and PARylation for SOX2-induced iPSC reprogramming, two recent studies have reported that ARTD1 and PARylation influence SOX2 in embryonic stem cells (ES cells)[76,149]. In particular, Gao and colleagues showed that in ES cells SOX2 represses *Fgf4* transcription by binding to *Fgf4*-enhancer and that ARTD1 ADP-ribosylates SOX2 to induce its eviction from the *Fgf4*-enhancer subsequently promoting *Fgf4*-gene transcription[76]. Conversely, in the second study, Lai and colleagues have shown that during retinoic acid-induced ES cells differentiation to neuronal precursors, ARTD1 PARylates itself but not SOX2[149]. Modified ARTD1 formed a complex with SOX2 to prevent SOX2 from binding to Sox2/Oct4-binding sites and repressed gene transcription[149]. Differences in the chromatin composition or in the signaling cascade may account for the divergent mechanisms of ARTD1 observed in reprogramming fibroblast or ESCs. In addition, to assess the role of ADP-ribosylation on gene transcription, both Gao et al. and Lai et al. used PJ34, a second generation inhibitor of ADP-ribosylation known to unspecifically target Pim1 and Pim2, 2 serine/threonine kinases that modulate gene transcription[126]. Therefore, PJ34 treatment may have induced changes in gene expression that were independent or only partially dependent on ADP-ribosylation inhibition. Instead, we characterized the effect of ARTD1 activity on gene transcription by treating the cells with ABT-888 a third generation and more specific inhibitor, limiting possible off-target effects[150]. Unfortunately, the lack of a specific anti-PAR antibody suitable for ChIP prevented us from showing that PAR is present at the *Fgf4*-enhancer together with SOX2. In fact, we cannot exclude that ARTD1 and PAR regulate SOX2 retention indirectly, without being physically associated with the *Fgf4*-enhancer. For example, ARTD1 and PARylated SOX2 may regulate the expression of genes other than *Fgf4*, whose protein products may be involved in recruiting SOX2 to *Fgf4*-enhancer in a PAR independent fashion. Thus, further ChIP analysis of ARTD1 and ChAP (see next paragraph) are needed to unequivocally prove the role of PAR in keeping SOX2 recruited to the *Fgf4*-enhancer. In addition, although we provided very strong evidence that SOX2 is ADP-ribosylated, SOX2 mutagenesis experiments are needed to elucidate what residues and domains are

targeted by PARylation and how this influences SOX2 properties. Moreover, it remains to be elucidated by which process the enzymatic activity of ARTD1 is induced during reprogramming. Interestingly, Lai and colleagues showed that the treatment of ESCs with exogenous FGF4 activated the FGF/ERK-signaling pathway leading to ARTD1 activation[149]. In fibroblast, *Fgf4* is not expressed and cannot be responsible for the activation of ARTD1[151]. However, we showed that *Fgf4* is transcribed in the initial phase of reprograming and that FGF4 induces fibroblast growth factor receptor (FGFR) signaling to promote iPS colony formation. Therefore, it would be interesting to address the effect of FGF4 signaling on ARTD1 activity. In this regard, Thomas et al. have recently shown that the Drosophila tandem kinase JIL1 phosphorylates histone H2Av at serine 137 to activate ARTD1 enzymatic activity[65]. JIL1 is the Drosophila orthologous of the mammalian mitogen- and stress-activated protein kinase 1 (MSK1) which, in response to mitogens signaling (e.g. FGF/ERK), phosphorylates serine 10 on histone H3 (H3S10ph) and other chromatin-associated proteins to promote gene transcription[152,153]. MSK1 activated by the FGF4 signaling in reprogrammed fibroblasts, might thus phosphorylate histone H3 serine 10 to subsequently activate ARTD1-mediated PAR-formation. If so, inhibition of the FGF/ERK-signaling or MSK1 depletion by siRNA should reduce reprograming-induced PAR formation and SOX2-dependent gene transcription. Comparing the transcriptomes of MSK1-depleted cells with ARTD1-/- cells would define the target genes that are simultaneously regulated by MSK1-signaling and PARylation. Furthermore, confirming the phosphorylation of histone H3S10 by ChIP analysis for these target genes would point at an interplay between such histone modification and PARylation.

Another recent study has shown that at early stage of reprogramming, ARTD1 and the ten-eleven translocation-2 (Tet2) cooperate to erase the DNA methylation at the pluripotent genes *Nanog* and *Esrrb* and promote their accessibility for the pluripotent transcription factor OCT4[154]. The authors describe a mere correlation between ARTD1 deficiency and 5-methyl cytosine (5meC) levels at these two pluripotent gene promoters, without providing further mechanistic insights. In addition, it is not clear whether ARTD1 enzymatic activity is necessary for 5meC erasure at these genomic sites. It remains open whether the ARTD1-mediated *Fgf4* transcriptional regulation described by our group is connected with ARTD1-mediated 5meC reduction. 5meC analysis of the *Fgf4* promoter and of the two analyzed *Nanog* and *Esrrb* promoters in

ARTD1-/- fibroblasts reprogramming would provide additional evidence whether demethylation can also be observed at other sites and whether demethylation is regulated by ARTD1. Moreover, complementation of these experiments with exogenous FGF4 would help to address whether FGF-signaling is responsible for erasing 5meC at *Nanog* and *Esrrb* promoters and for increasing OCT4 recruitment to these sites.

Our study raises some additional open questions that are worth to investigate. ARTD1 and its enzymatic activity might also influence additional processes later during iPSC generation. Given that ARTD1 and ADP-ribosylation regulate cell cycle at several steps[146] and considering the importance of cell proliferation in reprograming[147], it would be very interesting to study whether ARTD1 ablation or inhibition of ADP-ribosylation lead to additional changes in cell proliferation or cell cycling during the early-mid phases of reprograming[155,156]. Indeed, although Doege et al. have shown that ARTD1 overexpression does not alter cell proliferation in reprogramming cells, as measured by Br-dU incorporation, the effects of ARTD1 ablation or inhibition on cell proliferation has not yet been meticulously studied[154].

As ADP-ribosylation inhibited fibroblasts generate fewer iPS colonies when compared to ARTD1-/- fibroblasts, ARTD1 may not be the only ARTD generating PAR that contributes to fibroblast reprogramming. This could be addressed by quantifying whether ARTD1-/- fibroblasts treated with ADP-ribosylation inhibitor generate fewer iPS colonies than ARTD1-/- fibroblast. In particular, nuclear ARTD2 and its activity may directly contribute to chromatin remodeling[157]. Furthermore, ARTD5 or 6, as cytoplasm/nucleus shuttling poly-ARTDs, may be implicated in the cell signaling that leads to reprogramming and have an indirect effect on this process[158-160]. Thus, performing a siRNA screening including the above mentioned ARTDs and combining it with ChIP and gene expression analysis would help define the contribution of each member. In addition, while we have addressed the contribution of ARTD1-induced PARylation in reprograming, the role of protein MARylation needs still to be elucidated and additional efforts in this direction may reveal a far more complex network of modifications.

Cell reprogramming is emerging as a promising field in regenerative medicine. In order to redefine its identity, a somatic cell has to overcome some epigenetic barriers and express genes that in somatic cells are usually silenced. Here, we have reported that ARTD1-mediated ADP-ribosylation is part of the complex mechanisms that lead

to fibroblasts reprogramming. A complete understanding of the signaling that activates ARTD1 and promotes SOX2 ADP-ribosylation may lead to changes in the strategy currently used to generate iPS cells. For example, linking ADP-ribosylation to a specific signaling pathway may provide a way to activate ARTD1 in a temporal specific manner and increase the reprograming efficiency. In addition, an intriguing possibility is that ARTD1 over-expression or its targeted activation may replace one or more of the Yamanaka oncogenic factors limiting the risk of tumorigenesis. Furthermore, as ADP-ribosylation has been implicated in both reprogramming and cell differentiation, changing the pattern of ADP-ribosylation in a somatic cell, through a combination of PAR-inducing stimuli, may favor transdifferentiation bypassing the need for a pluripotent intermediate.

4.2 ADP-ribose binders as tools to investigate chromatin-associated protein ADP-ribosylation

In this second part of my thesis I describe a novel chromatin affinity precipitation (ChAP) method that allows to further analyze the nuclear dynamics of chromatin ADP-ribosylation. By applying this method to investigate H_2O_2-mediated ADP-ribosylation we have showed that it preferentially localized to nucleosome-dense and heterochromatic regions and to repetitive elements within the genomic DNA. Conversely, during adipogenesis, ADP-ribosylation was associated with PPARγ on PPARγ target genes confirming some previous finding of our laboratory[92].

In vitro observations have indicated that ARTD1-mediated PARylation of histones and other chromatin-associated proteins increase their negative charge repelling them from the DNA and inducing chromatin de-compaction[161]. *In vivo* studies on the *hsp70* promoter in the salivary glands of Drosophila confirmed that ARTD1 auto-modification and trans-ADP-ribosylation of histones induces chromatin loosening and puff formation to enhance *hsp90* gene transcription[162]. A similar mechanism has also been reported to regulate a subset of NF-κB target genes in murine macrophages, although ADP-ribosylation in this study might have been induced during cell lyses [64]. Our data indicated that H_2O_2-induced ADP-ribosylation did not provoke ARTD1 or nucleosome eviction, but increased the accessibility to the DNA in the chromatin context.

To study protein and protein-PTM localization on the genome, ChIP is the most commonly used technique in molecular biology. For studying chromatin-associated

ADP-ribosylation, this approach has been limited by the lack of ChIP-specific antibodies. Indeed, 10H, the most common anti-PAR antibody, showed unspecific binding to chromatin when used for ChIP, possibly due to the repulsion of proteins from chromatin due to long PAR-chains, the epitopes of 10H antibody[55,57]. Similar results were obtained with another commercially available polyclonal anti-PAR antibody, suggesting that anti-PAR antibodies are not suitable for ChIP under the tested conditions. Therefore, we functionally replaced the anti-PAR antibody with a GST-tagged WWE domain, a domain with high affinity for iso-ADP-ribose, the smallest PAR structure, and developed a ChAP method for enriching endogenously ADP-ribosylated proteins in the chromatin context. A similar strategy has been already applied in two previous studies where the authors used the macrodomain Af1521, an ADP-ribose binder that recognize both MARylated and PARylated proteins, combined with mass spectrometry (MS) to identify ADP-ribosylated proteins non fixed to chromatin [57,58]. While in the first study Dani et al. identified only few proteins to be ADP-ribosylated in untreated cells, Jungmichel et al. expanded the analysis by treating HeLa cells with different genotoxic stimuli, which led to the identification of 235 ADP-ribosylated proteins[57,58]. Most of the identified proteins localized to the nucleus and were involved in DNA and RNA metabolism. However, due to the cell lysis, none of the above studies addressed the ADP-ribosylation of chromatin-associated proteins. Moreover, very little is known about the localization of ADP-ribosylated chromatin-associated proteins within the nucleus. For example, heavy PARylation of ARTD1 or nucleosomes has been reported to repulse proteins from the chromatin, whereas some transcription factors, once PARylated, increase their affinity for the DNA[64-66,138,139]. Our ChAP data indicate that, after induction of protein ADP-ribosylation by H_2O_2, the modified proteins remain associated with the DNA, under the testes condition. However, we cannot exclude that the extent of ADP-ribosylation might differ between the modified proteins and that only proteins modified with short PAR could be detected by ChAP whereas heavily modified proteins were repulsed from the chromatin. Alternatively, the PAR glycohydrolase (PARG) and other PAR-degrading enzymes may counteract ARTD1 activation and keep the PAR length below a certain length preventing proteins from being evicted from DNA and allowing the detection of the whole chromatin-associated ADP-ribosylome[25].

Our genome-wide analysis of ADP-ribosylated protein revealed that H_2O_2 induced chromatin ADP-ribosylation is not randomly distributed but rather targeted to repetitive sequences such as LINEs, SINEs and α-satellites in intergenic regions or in introns but not in exons. These results indicate that there is a local discrimination in the chromatin that prevents coding sequences from being ADP-ribosylated and favors instead latent sequences. How this discrimination is established remains so far unknown. One possibility is that ARTD1 is more enriched at repetitive sequences (compared to transcriptionally active sequences) and therefore these regions are more prone to be ADP-ribosylated in response to H_2O_2. A genome-wide profiling of ARTD1 occupancy by ChIP-Seq would help to understand whether ARTD1 and the herein detected PARylated protein peaks overlap. Unfortunately, all so far available genome-wide ARTD1 analyses focused on promoter regions[68,163]. In line with that, another open question is, what regulates the recruitment of ARTD1 to the chromatin. Although some reports claimed to have identified an ARTD1 DNA-consensus sequence, more studies are needed to validate these findings[164]. A sequence and structural analysis of the genomic loci associated with PARylated domains may reveal common features that may be involved in ARTD1 recruitment. Indeed, it has been reported that ARTD1 has binding preferences for structured DNA[165]. In addition, as we provide evidence that ARTD1 occupancy strongly correlates with histone H3 occupancy, nucleosomes might be a docking platform for ARTD1 (see below). Thus, ChIP and ChAP-ChIP analysis of ARTD1, nucleosome, histone variants and histone modifications at the identified ADP-ribosylated chromatin regions would help to define the chromatin context necessary to bind and activate ARTD1 in response to H_2O_2. In addition, defining whether ADP-ribosylated repetitive elements are associated with transcribed genes and whether they generate transcripts may help to address any involvement of RNA in ARTD1 activation.

The observation that protein ADP-ribosylation preferentially localized at nucleosome dense regions and repetitive sequences was surprising, as H_2O_2 induced ADP-ribosylation was thought to occur mainly on open chromatin, as it should be more exposed to DNA oxidation[129]. This may indicate that H_2O_2 induces DNA damage not randomly but mainly at genomic loci with high nucleosome content and repetitive elements where ARTD1 is then activated to initiate DNA repair. Extensive DNA damage is known to induce an over-production of ADP-ribosylation that depletes the cellular ATP pool and induces cell death by necrosis[166]. In this context, chromatin

ADP-ribosylation may then be a temporary storage of intracellular energy in a form that can be reused by the subsequent action of PARG and ADP-ribose pyrophosphorylase to regenerate ATP[167]. However, by doing so, the ADP-ribosylated chromatin regions become more accessible and potentially more exposed to mutagenesis, which is the down side of this process. Therefore, targeting ADP-ribosylation to chromatin with repetitive DNA sequences may be an elegant way exploited by the cell to store energy at non-expressed genomic regions preventing coding regions, regulatory elements and non-coding RNAs from being exposed and harmed by possible mutations.

Our laboratory recently published that during differentiation of 3T3L1 to adipocytes, topoisomerase II activity induces ARTD1-auto-modification and thereby sustains PPARγ target genes transcription[92]. Our newly developed ChAP method allowed us to enrich chromatin fragments ADP-ribosylated during adipogenesis and to confirm that ADP-ribosylation regulates PPARγ target-genes in a way similar to what happens during reprogramming at SOX2 target-genes. ARTD1 generates PAR to keep PPARγ in close proximity to its DNA response elements, preventing it from dissociating from the DNA and thus increasing PPARγ-dependent gene transcription.

Together, the newly developed ChAP method represents a powerful tool to study the interplay between ADP-ribosylation and other histone modifications in the chromatin context in different cell types. The interplay will most likely define how ADP-ribosylation is induced and how it influences the chromatin structure and the functional implications in chromatin remodeling, DNA repair or gene transcription. Within a certain cellular process, the ChAP technique will help researchers to discriminate between those genomic loci or genes that are directly regulated by ADP-ribosylation and those that are indirectly affected, a discrimination that is not evident by using ADP-ribosylation inhibitors. Given the great medical relevance of ADP-ribosylation inhibitors in the treatment of certain cancers, in modulating inflammation and in cellular reprogramming, the ChAP method will provide scientists with a tool to further dissect the molecular mechanisms underlying the promising effects of ADP-ribosylation inhibitors.

4.3 Effect of ADP-ribosylation inhibitors on NF-κB-dependent gene expression

One of the aims of this thesis was to define the contribution of ADP-ribosylation on NF-κB activation and target gene transcription. Since previous studies showed that inhibition of ADP-ribosylation had a beneficial effect on LPS-induced endotoxic shock in mice, and since ARTD1 has been implicated in NF-κB target gene transcription, we hypothesized that ADP-ribosylation might directly contribute to NF-κB-induced gene activation[168,169]. However, when we induced NF-κB in A549 cell with pro-inflammatory compounds, we did not observe any effect of PARylation on gene transcription, although only IL1β and IFNγ combined with TNFα were able to successfully induce NF-κB-dependent gene expression. Similar data were obtained in a macrophage cell line (personal communication of Dr Minotti) in our laboratory, suggesting that either PARylation does not affect NF-κB target gene transcription or that it regulates other NF-κB target genes not taken in account in our analysis, or that the beneficial effect of the ADP-ribosylation inhibitors during inflammation *in vivo* is due to inhibition of ARTD-members that are not inhibited by ABT-888[170]. In our analysis we restricted the cell stimulation to a single NF-κB-activating compound whereas *in vivo* cells are exposed to a number of pro-inflammatory stimuli that may account for the activation of ADP-ribosylation. In addition, the in vivo generation of ADP-ribosylation may not be due to NF-κB activation, but rather due to cellular stress as side effect, for example, of high body temperature or cell exposure to toxic molecules released by other necrotic cells. Expanding the analysis using a combination of several pro-inflammatory compounds and different temperature and performing a deep-sequencing RNA analysis on stimulated cells may reveal the molecular mechanisms underlying the beneficial effects of inhibition of ADP-ribosylation during inflammation.

To address the mechanisms underlying the beneficial role of ADP-ribosylation inhibition in animals challenged with pro-inflammatory stimuli, *in vitro* models may nor represent a good option. A systematic approach in animal models is necessary to address the molecular effect of ADP-ribosylation during acute or chronic inflammation. In addition it may be useful to develop a rapid and automated “in cell” sensor that can be used to detect the formation of ADP-ribosylation. In this way

different treatments could be tested to address what combinations of factors are indeed responsible for the formation of ADP-ribosylation in cells.

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